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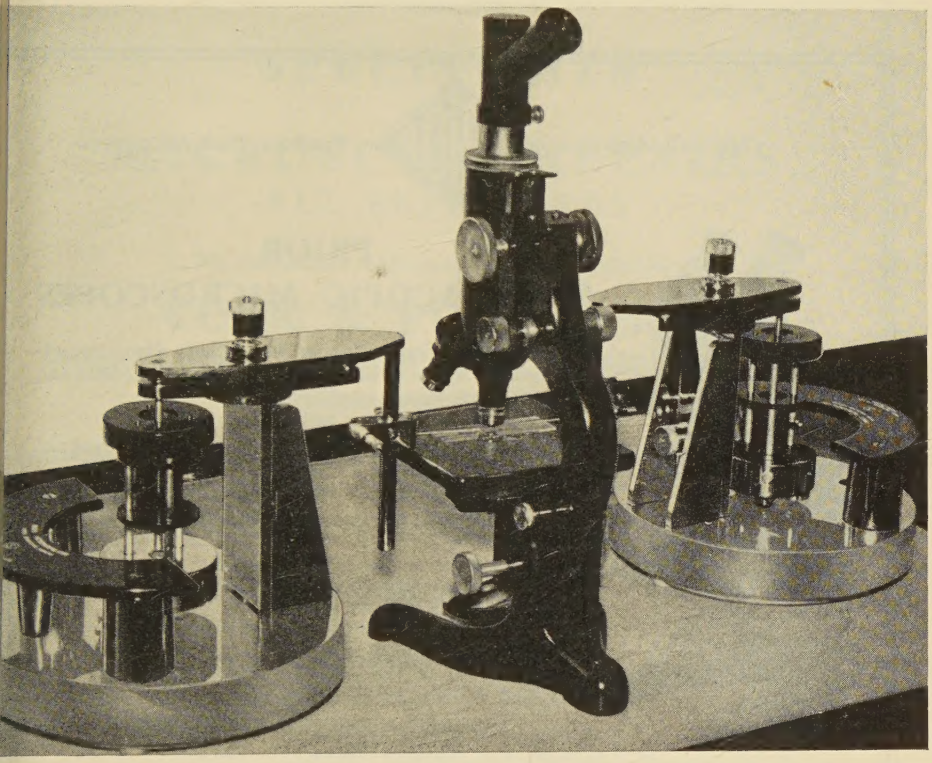

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Experiments on the Fixation of Lipids by Osmium Tetroxide

By J. T. Y. CHOU

(*From the Cytological Laboratory, Department of Zoology, University Museum, Oxford*)

Summary

The black material seen in paraffin preparations of tissues fixed with osmium roxide is not merely reduced osmium. The lipid is still present in combination th osmium. Osmicated lipid globules are insoluble in chloroform or benzene. ney regain their solubility in lipid solvents when the blackness caused by osmium roxide is bleached away.

Introduction

N paraffin sections of tissues fixed with osmium tetroxide, the lipid-sites are often black. The purpose of the present investigation was to find nether the lipids are still present in these lipid-sites or whether they have en dissolved away by the antemedium before the tissue was embedded in e paraffin wax, leaving only a black residue.

Materials and Methods

The skin of the mouse and the liver of the newt were used for this vestigation.

The skin, shaved with a razor-blade, and the liver were cut into small eces and fixed for 24 h either in Flemming's fluid or in 1% osmium roxide.

A standardized process of dehydration was used throughout. After the ssue had been washed for 24 h in running water, it was left for $\frac{1}{2}$ h in each the following grades of ethanol: 50%, 70%, 80%, 90%, 95%, and absolute. issue was first left in a mixture of equal volumes of absolute ethanol and temedium for $\frac{1}{2}$ h and then in the antemedium alone for the same period ne fluid was changed once). The antemedia used were chloroform, benzene, luene, and xylene. After being soaked in one of these antemedia for $\frac{1}{2}$ h, e tissue was transferred to melted paraffin wax (m.p. 56° C) and left for 1 h ith one change of wax).

Sections of the skin of the mouse were cut at 15 μ and those of the liver of e newt at 8 μ. All sections were dewaxed in the fluids previously used as temedia.

The sections were examined under the microscope while still in water and en bleached in 3% hydrogen peroxide solution. The period of bleaching ried; but after bleaching, the sections were always examined under the croscope to make sure that the blackness had been removed. The sections re then coloured for 5 min in a saturated solution of Sudan black B in 70% anol. After coloration, the sections were rinsed for 5 sec in 70% ethanol

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and 1 min in 50% ethanol; they were washed in distilled water and mounted in Farrants's medium.

To study the solubility of osmicated lipid globules in lipid solvents other than the antemedia already mentioned, acetone and pyridine were also used. The skin of the mouse was fixed in Flemming's fluid, dehydrated, and passed through benzene into paraffin. Sections were dewaxed in benzene and transferred to cold acetone at room temperature (about 20° C) for 24 h; or, alternatively, sections were dewaxed in benzene and treated in boiling acetone for the same period. The sections were brought to water and bleached by the method described above; they were then coloured with Sudan black.

Pyridine was used in a similar way. After dewaxing, sections were transferred to pyridine either at room temperature for 24 h or at 60° C for 19 h. They were then coloured with Sudan black.

Results

The lipids of both kinds of cell were blackened by fixation in osmium tetroxide solution. When the sections were left in the antemedia for 5 min to

Table 1

The reaction between the different antemedia and lipid globules

Period of dewaxing at room temp. (20° *C*) (*min*)	*Antemedium and dewaxing agent*			
	Xylene	*Toluene*	*Chloroform*	*Benzene*
5	+	+	+	+
40	O	O	+	+

+ indicates coloration by Sudan black.
O indicates that no sudanophil material was present.

remove the wax, the lipid-sites were still black. If, however, the sections were left in xylene or toluene for 40 min instead of 5 min, the lipid-sites were colourless, and usually appeared empty. They remained black, however, if benzene or chloroform was used for the same period.

When sections that had been 5 min in any of the antemedia were brought down to water and bleached, the lipid could easily be coloured by Sudan black. If sections were left for 40 min in the antemedia, the ones treated with xylene or toluene were not coloured by Sudan black (see table 1). This showed that there was no lipid in them. However, those that had been treated in benzene or chloroform for 40 min showed a positive result.

The lipid globules in the skin of the mouse sometimes showed a network like structure, though they were black all through before the bleaching reagent was used; sometimes, however, these globules were homogeneously coloured by Sudan black. The lipid globules of the liver of the newt sometimes showed as a thick, black cortex, but in most cases they were homogeneously blue black with Sudan black.

Sections were bleached with hydrogen peroxide, taken up to the same temedia in which they had been dewaxed, left there for 5 min, and then ought down to 70% ethanol; eventually they were coloured in the usual y with Sudan black. The result was in all cases negative. This showed that lipid-sites no longer contained lipids. The same results were obtained ether the antemedium was xylene, benzene, toluene, or chloroform.

From the above experiments the conclusion can be drawn that so long the lipid-sites were still black, they contained lipid. The black substance ust be a compound of lipid with osmium. This compound is insoluble in nzene or chloroform, but dissolves slowly in xylene or toluene; lipid is set e by bleaching.

When sections that had been dewaxed in benzene were treated with cold boiling acetone, the osmicated lipid globules usually remained black all rough, and could be blackened by Sudan black after bleaching. Sometimes, wever, a certain amount of solution by acetone occurred, and the Sudan ack was then distributed in the form of a network.

In sections treated with cold pyridine and bleached, the sudanophil aterial was in the form of a network. It is evident that partial solution by cold ridine had occurred. After treatment with pyridine at 60° C, no osmicated ids remained in the lipid-sites.

I wish to acknowledge the help I have received from Dr. J. R. Baker, R.S. Miss S. H. Macpherson kindly repeated some of the experiments.

The Action of Colchicine on the Intestinal Epithelium of the Cat and Dog

By R. M. H. McMINN

(*From the Department of Anatomy, University of Sheffield*)

Summary

Varying doses of colchicine have been administered intraperitoneally to cats and dogs in order to study histologically the reactions in the epithelium of the small intestine. In normal animals of both species, approximately 1% of the total number of epithelial cells in the ileum are in mitosis at a given time. After exposure to colchicine for 5 h, the maximum number of arrested mitoses in the cat was found to be 3·77%; in the dog the maximum number rose to 17·12%. The results indicate that in the epithelial cells of the dog's small intestine, colchicine not only arrests mitosis in metaphase, but also stimulates the cells to enter mitosis.

Introduction

THE use of colchicine as a mitotic inhibitor has been widespread on the assumption that, with suitable dosage, cells that have begun to divide will be arrested in the metaphase stage of the mitotic cycle. As a result of this arrest, if an animal is killed a few hours after the administration of the drug, an accumulation of arrested metaphases will be seen, representing the number of cells that would normally have gone on to complete their division during that time. This effect is produced by interference with the formation of the spindle (Ludford, 1936; Hughes, 1950). In a recent study with the electron microscope, Inoué (1952) concluded that the action of colchicine on the spindle is 'to disorganize the orientation of the micelles in the astral rays and spindle fibers'. The assumption that any metaphase seen a few hours after colchicine administration is that of a nucleus that would normally have undergone mitosis and that has not been artificially stimulated to do so, is the basis of many biological studies. With some exceptions, its use has been confined almost entirely to experiments on mice, rats, and embryos. Little information is available regarding mitotic inhibition by this substance in larger animals. In the course of studies on the renewal and regeneration of epithelium in the small intestine of the cat (McMinn, 1954; McMinn and Mitchell, 1954), colchicine was used to arrest mitosis among the cells lining the intestinal glands (crypts of Lieberkühn). The dose employed in those studies was determined by a small series of pilot experiments, since no previous reports on the use of colchicine in the cat were available. Those experiments have been extended to include a similar series in the dog. The results of this work are now presented, since they have revealed a fundamental difference between the two species with regard to the reaction of the intestinal epithelium to colchicine.

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Materials and Methods

All experiments were performed on healthy adult cats and dogs. The animals of each species were divided into 5 groups of 4 animals in each group. One group served as normal controls, and the remaining groups received doses of colchicine (see table 1), administered intraperitoneally at 10 a.m. The animals of all groups had been starved since the previous evening and all were killed at 3 p.m. Possible discrepancies due to diurnal mitotic variations were thus eliminated. The histological details and the method of enumerating the epithelial nuclei were the same as those previously reported (McMinn, 1954). Briefly, the mitotic counts were carried out on specimens of ileum (about 50 cm proximal to the ileo-colic junction) stained with haematoxylin and eosin. A square mask was used in one of the eyepieces of a binocular microscope. At least 2,000 nuclei were included in any single count, 4 such counts being carried out in each animal. Although mitosis occurs only in the cells lining the crypts and not in those covering the villi, the number of dividing nuclei is expressed as a percentage of the total number of epithelial nuclei in both crypts and villi. A number of duodenal specimens were also examined, but since the results were similar to those in the ileum no further reference is made to them.

Results

The results of the mitotic counts in both the normal and colchicine-treated animals are summarized in table 1, the percentages being the means of the counts in the 4 animals of each group.

Table 1

			Anaphases and telophases	*Percentages of nuclei in mitosis or arrested mitosis (means ± S.E.)*
cat	normal		present	0·95±0·04
	colchicine	(mg per kg) 0·1	present	1·27±0·30
		0·25	absent	3·77±0·33
		0·5	absent	1·98±0·08
		1·0	absent	1·62±0·12
dog	normal		present	1·03±0·34
	colchicine	0·05	present	2·51±0·25
		0·10	present	2·82±0·23
		0·15	absent	7·69±0·43
		0·25	absent	17·12±1·00

From the table it can be seen that in the normal cat and dog approximately 1% of the total number of epithelial cells were in mitosis at any one time. In the cat, colchicine in a dose of 0·1 mg per kg was insufficient to cause complete arrest in metaphase of all dividing nuclei, since a considerable number of

nuclei in later phases of mitosis were still found. With larger doses, anaphases and telophases were no longer seen, and the maximum percentage of mitotic nuclei was observed with 0·25 mg per kg. Higher doses resulted in a percentage of arrested nuclei that was not significantly greater than that in the group of normal controls.

In the dog it was found necessary to give a range of smaller doses, since with 0·25 mg per kg (the highest dose given to a dog) severe toxic signs developed, such as were seen in cats receiving 1·0 mg per kg. The number of arrested nuclei found in dogs to which 0·25 mg per kg had been administered reached 17·12% of the total number of epithelial nuclei. Doses of 0·05 and 0·1 mg per kg were insufficient to cause complete metaphase arrest, while 0·15 mg per kg gave rise to nearly 8 times as many mitotic cells as in the normal group, and no later phases of the mitotic cycle were evident.

Thus the maximum percentage of dividing nuclei in the cat—3·77% after exposure of 5 h to a dose of 0·25 mg per kg, which was not the largest tolerated dose—was less than 4 times that found in the normal animal. However, in the dog, which displayed a normal figure similar to that in the cat (about 1%), the greatest number of arrested nuclei, 17·12%, was found after administering the largest tolerated dose of the series.

Discussion

The results indicate the pronounced difference that exists between the cat and dog with regard to the reaction of intestinal epithelium to colchicine. The fact that the cat (of about 2 kg) seems to have a greater tolerance for colchicine than the dog (which is considerably heavier), is in keeping with the tolerance shown by the mouse, which may weigh 25 g and receive a total dose of 0·1 mg, i.e. at a rate of 4 mg per kg (Bullough, 1949), compared with the rat, for which the dose usually suggested is 1 mg per kg (Bertalanffy and Leblond, 1953; Ebling, 1954).

In the cat, doses exceeding 0·25 mg per kg do not result in a continuing increase in the number of arrested nuclei. This suggests not only that nuclei are being arrested in metaphase but also that some are being prevented from entering mitosis. Brues and Cohen (1936) and Bucher (1939) noted that doses larger than those required to give a maximal response normally caused a diminution in the number of arrested nuclei. A similar phenomenon was noted by Ebling (1954) in rat skin, and by Henry, Meyer, Weinmann, and Schour (1952) in the oral epithelium of rabbits. Bullough (1949) concluded that after about 5 h resting cells (in the ear epidermis of the mouse) were prevented from entering prophase. However, in the dog, increasing the dose increased the number of arrested nuclei. The maximum percentages found in the cat and dog are 3·77 and 17·12 respectively, after an equal period of exposure to colchicine. Since in normal animals of both species only about 1% of cells are in mitosis at a given time, it would appear that in the dog, cells are being stimulated to enter mitosis, on the assumption that the duration of the mitotic cycle is approximately the same in both species.

This apparent stimulation of mitosis by colchicine is a feature of its action that was recognized as early as 1906 by Dixon, who stated that its effect was to 'excite karyokinesis'. Paff (1939) considered that in the chick embryo colchicine could stimulate as well as inhibit cells that were rapidly dividing. In more recent experiments on tadpoles, Crișan and Mihalca (1948) were of the opinion that colchicine was stimulating cells that were about to divide. In contrast to these observations, current views on the action of colchicine have been summarized by Miszurski and Doljanski (1949), who stated: 'it is now always taken for granted that colchicine has no positive or negative effect on the rate of appearance of the mitoses before they are arrested and that its only effect is to arrest cell division that is already in progress'. Evidence in support of this is provided by the tissue culture studies on both normal and malignant cells reported by Ludford (1936), Bucher (1939), Törö and Vadász (1939), Tennant and Liebow (1940), and Hughes (1950), while in the living animal the work of Brues (1936), Buschke, Friedenwald, and Fleischmann (1943), and Bullough (1949) may be cited.

While most investigators who have used colchicine as a mitotic inhibitor have employed relatively short time intervals, others have thrown further light upon the possible stimulating action of this substance by studying the delayed effects following a single or repeated injections. Miszurski and Doljanski (1949) found an outbreak of mitotic activity in the rat's liver (where mitoses are normally uncommon) that was maximal 3 days after the injection, while Dustin (1941) distinguished between the mitoses that are seen within a few hours of injection in tissues with relatively high mitotic indices, and the 'mitoses tardives' that are found after a period of several days in various organs in which mitotic activity is normally very low.

Although the results of the present work are an indication that in the dog colchicine can stimulate the cells of intestinal epithelium to enter mitosis, it remains to be seen whether other tissues in this species exhibit a similar behaviour. The present findings have substantiated the dose used in the cat in previous reports (McMinn, 1954; McMinn and Mitchell, 1954), but have shown that it is not possible to calculate the rate of renewal of intestinal epithelium in the dog by use of the colchicine technique. They serve to emphasize the importance of carrying out studies with varying doses of colchicine, in species whose reaction to the drug is not well established, before its proposed use (for example) in the estimation of the rate of renewal of cell populations (Leblond and Walker, 1956).

I am indebted to Professor Francis Davies for his helpful criticism during the preparation of this paper, and to Messrs. J. H. Kugler and J. H. Morrill for technical assistance.

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Some Cytological Features of Epididymal Cells in the Rat

By B. L. REID

(*From the Department of Histology and Embryology, University of Sydney*)

With three plates (figs. 1–3)

Summary

Living material was photographed by phase-contrast miscroscopy. Fixed material was stained for mitochondria or post-osmicated.

The findings show a close conformity between the pictures in living and fixed cells, except that the so-called Golgi apparatus, whilst very apparent in some zones by both techniques, in other zones is well represented after osmication but not in the living cell. Reasons for this are proposed.

Studies by earlier workers on the mitochondrial pattern are confirmed. There is an increased density and decreased length of rods down the length of the epididymal duct. The relationship of the chondriome to the osmiophil material varies in the different zones; in some, close association with permeation is found, while in others the separation is complete.

General observations upon living epididymal cells are recorded. These include the effects of different suspending media and the changes undergone by the cells after their isolation. A close study has been made of the free (stereociliated) border of the cells.

Contents

Introduction

IT became apparent in previous studies of the epididymis (Reid and Cleland, 1957) that there were marked differences in the appearance of the cells in the different zones of the epididymal duct of the rat. In the present communication these histological differences have been given a cytological interpretation. The more conspicuous of these, Golgi apparatus and chondriome, have been closely studied in both living and fixed material, and a communication devoted solely to these structures is considered both a necessary extension to the histological studies and an essential prerequisite to an investigation of the detailed physiology of the organ.

Most of the early cytological work on the rodent epididymis was done on the mouse, occasionally on the guinea-pig, but rarely on the rat. Nassonov (1924) credits Negri with the first description of the Golgi apparatus in gland

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cells (organ not specified) by the silver impregnation method. Aigner (1900), who studied the rat epididymis, described the vacuolated area (probably analogous with the Golgi area) of zone I C or II, the fine granules of the isthmus region, as well as the juxtanuclear vacuoles of zone 4 A and B. Regaud (1901) described the granules present throughout the rat epididymis and the variations in nuclear shape. Fuchs (1902) certainly recognized the Golgi structure in the mouse epididymis but always refers to it as *Fadenknäuel*, his figures showing it in contact with the cell surface by a bundle of filaments and so discharging its secretion. Holmgren's trophospongium is undoubtedly the same area.

Benoit (1926) described the cytology of the rat epididymis. He observed the abundance of the mitochondria in body and tail and their invasion of the Golgi region in these segments. By contrast, in the mouse, he noted the relatively poorly developed mitochondria of the epithelium of this initial segment and their filamentous shape, and implies that here they do not invade the Golgi region. Benoit also homologized the trophospongium of Holmgren with the *Fadenknäuel* of Fuchs and gave an accurate description of the disposition of this material in the zones which he distinguished, viz. elongated in the *segment initiale*, rounded in the end of the head region, flattened in the body, and compactly globular in the tail. In the same paper he described the scant mitochondria and Golgi apparatus of the basal cells.

Nassonov (1924), Ludford (1925), and Parat (1928) have all contributed to the Golgi and mitochondrial picture in the mouse and more recently Laurent (1932) and Mietkiewski (1935) have studied these structures in the guinea-pig. Using more recent techniques Dalton and Felix (1953, 1954) have directed attention to the Golgi apparatus of the mouse as shown by phase-contrast and electron microscopy. Christie (1955) noted the mitochondrial pattern and described a 'supranuclear' body of osmiophil material in the mouse epididymal cell.

Throughout this paper the author has used the term 'Golgi apparatus' as applying to that osmiophil organelle more generally described as 'apparatus' or 'area', in the full knowledge that the justification for so grouping what is probably a heterogenous complex with doubtful inter-tissue homologies has only the sanction of usage and convenience.

Similarly the word 'chondriome' has been used generically to cover those cytoplasmic inclusions, including rods, rodlets, and granules, which do not allow further subdivision into mitochondrion, secretion granule, &c., from their appearances in living cells.

Materials and Methods

Fresh and fixed material has been studied. In the fixed material, small pieces of tissue dissected from the appropriate anatomical region were fixed either in Mann's fluid, which was followed by Ludford's (1926) modification of the Mann–Kopsch technique, and in Helly's fluid, followed by postchroming (48 h at 37° C in 2·5% $K_2Cr_2O_7$) and staining by the Bensley–Cowdry

Cowdry, 1918) or Kull (1914) procedures. Blocks were embedded in paraffin and sectioned at 3 μ.

For fresh preparations similar small pieces were usually suspended in homologous serum charged with carbon dioxide and kept at 35° C, but in one experiment isotonic sucrose was used. They were then minced with scissors and teased with fine needles upon siliconed slides and promptly examined by phase-contrast microscopy. For an average preparation these manipulations were completed in 15 min after death.

Results

Description of the micro-anatomy of the epididymis in these observations will conform to that proposed by Reid and Cleland (1957). A short summary of the principal histological features will precede each section.

Efferent ducts

The efferent duct is divisible histologically into two zones: (i) an initial zone characterized by a fairly tall epithelium with basal nuclei. The nuclei are smooth in contour with fine chromatin material. The cytoplasm is characterized by a finely vacuolated region occupying $\frac{1}{3}$ to $\frac{3}{4}$ of the supranuclear area, occasional large granules just beneath the surface, and a free border beset with brush or apocrine cupula terminalis; (ii) a terminal zone with absence of supranuclear vacuolation and with large numbers of small granules.

Both zones contain ciliated cells of varying height and shape with rounded nucleus and characteristic row of basal granules at the insertion of the cilia.

Initial zone. Fresh preparations of the initial zone confirm the vacuolated state of the cytoplasm. It is to be noted, however, that the living cell does not appear as translucent as it does in fixed preparations stained with eosin. Mitochondria are not clearly seen, but the cytoplasm contains granules scattered throughout the cell. The nature of these is uncertain; they have no obvious counterpart in stained material. The general appearance is of a featureless cell.

These preparations are not so informative as to the structure of the free border of the cell. Phase-contrast pictures merely show an accumulation of homogeneous structureless material whose border, both with adjacent cells and with lumen, is ill defined. This is, presumably, some secretion product, or it may be a brush border as seen at the limit of resolution in serum. Sections of material fixed in Helly sometimes show an ultrafine fringe at the free border about 5 μ tall.

Single-cell fresh preparations suggest a higher frequency of binucleate cells than was apparent in fixed tissue. In one sample of over 200 cells, 10% of the cells were binucleate.

Variation in the shape of ciliated cells as seen in histological preparations becomes very evident in single-cell preparations. Even from the same zone, initial or terminal, they vary from cuboidal through wine-glass (the shape usually figured) to elongate cylindrical. A tenuous extension at the cell base

in the shape of a 'foot' is also common. The basal granules are clear-cut. Mitochondria can be discerned throughout the cell and there are numerous granules especially about the apical pole of the nucleus in the form of a crescentic mass. Chance preparations show ciliary movement with an approximate frequency of 5 to 10 per sec.

In fixed preparations mitochondria were filamentous and evenly distributed throughout the cell, skirting the spaces which we have called vacuoles. Fig. 1, B shows this distribution.

Sections from Mann-Kopsch preparations show a rather special distribution of the Golgi apparatus. A collection of three or four osmiophil threads in randomly directed arrangement lie in the apical third of the cytoplasm only a short distance from the cell border. They surmount a featureless zone of cytoplasm immediately above the nucleus. The latter zone is devoid of cytoplasm apart from a few sparsely scattered granules (fig. 1, C). On the other hand, the ciliated cells are usually heavily blackened. The black material consists of a supranuclear 'stringy' or fibrillar mass of osmiophil substance and infranuclear lines of rodlets reminiscent of the arrangement of the mitochondria in these cells. In non-ciliated cells the osmium has no special affinity for mitochondria.

Terminal zone. In fresh preparations, the terminal zone of the efferent ducts is characterized by variations in cell size and shape ranging from spherical-cuboidal to elongate-columnar. A corresponding variation occurs in the ciliated cells. The general appearance of the cells is not greatly different from those in the initial zone. The vacuolation is not so marked, although the cells are beset with numerous granules irregularly placed about the cytoplasm, again bearing no relation to the fuchsinophil granules so conspicuous in this area in stained preparations. Narrow perinuclear spaces evident in some of these cells in fixed preparations are conspicuous in the fresh material. Fresh material does not help the understanding of the cell border. The cell here terminates merely in a structureless irregular coagulum.

In fixed preparations mitochondria are seen to be scattered throughout the cytoplasm, as in the initial zone. Pallisades of filaments occur beneath the free border of the cell and shorter plumper rods are found between the nucleus and the basement membrane.

The Golgi zone is not prominent, occurring at some distance above the nucleus although to a less pronounced degree than in the initial zone. It consists of a few interconnected strands of osmiophil material (fig. 1, D). Impregnation was never good. In no instance was there a great development of osmiophil material associated with chains of vacuoles, as seen elsewhere in the epididymal duct.

The obvious difference between living and fixed cells was the general opacity of the living cells of the initial zone, which do not appear as highly vacuolated as the fixed cells. The granules of the cells of the terminal zone, which are so conspicuous in sections stained with fuchsin, are not seen in living cells.

However, as regards other cytological details and cell shape there is a marked correspondence between the living and fixed cells.

pididymal duct

Zone I A and B is characterized by very tall principal cells with apical cells terposed. There is a separate, finely vacuolated, lightly staining region mediately above the nucleus, occupying the second quarter of the cyto- asm. Nuclei are smoothly contoured. From the apical surface project long, agile stereocilia.

Fresh preparations of dissections from zone I A and B were all charac- rized by optical homogeneity and low refractive index of nucleus and cyto- asm, caused largely by the absence of cell inclusions. These cells were haracteristically flexible, so that when seen flowing along in a stream they ould temporarily assume contorted shapes, only to revert to their tall olumnar shape when at rest. No such variation in shape was seen in cells om elsewhere in these ducts.

Favourable resultion of stereocilia showed them as a bundle of about o slender, immobile hairs, projecting in the long axis of the cell from the entral portion of the free surface for about $\frac{1}{3}$ to $\frac{1}{2}$ of the length of the cell. Mitochondria were often seen as long filaments lying haphazardly down the ngth of the cell but were most numerous in the inner half (with respect to he lumen). Apical cells were often found and mitochondria were conspicuous n them. The presence of the Golgi area was inconstant and this could not be elated to any other observation, e.g. the state of surrounding cells, the state f mitochondria, or the presence or absence of nucleoli. When present it took roughly spherical shape. It was separated from the nucleus by a thin strand f cytoplasm and composed mostly of rows of vacuoles peripherally enclosing n area of the same appearance as the remainder of the cytoplasm.

The nucleus in these cells has a refractive index nearly the same as that of he cytoplasm. When apparent, the nuclei lie at some distance from the base- ient membrane. In fresh preparations little can be seen of the nuclear mem- rane or nucleoli. Binucleate cells were seen just as is the case in the efferent uct cells.

Postchromed preparations for mitochondria confirmed the picture of the andomly orientated, long filaments seen in living cells. They lie mainly arallel to the length of the cell and are especially concentrated toward the pical half of the cell. The concentration referred to is relative only, since the itochondria of zones I A, B, and C are nowhere as dense as in more caudal ones. The Golgi area is free of mitochondria.

The fraction impregnated by osmium methods conforms to the vacuolated rea in the living cells and consists of a well-defined area on the luminal side of he nucleus about twice the length of the nucleus and separated from it by small strip of cytoplasm. Favourably impregnated areas of tissue show the smiophil material to be generally distributed over the supranuclear area; the eriphery of this area is vacuolated. The occasional apical cells which were

impregnated showed osmiophil material extending from the supranuclear position along one side of the nucleus into the pointed base of the cell. This extent does not correspond with the vacuolated area in the living cell.

With regard to specificity, the whole question of osmium impregnation of the epididymis is unsatisfactory. Certain zones always seem to impregnate well (e.g. zone I C and II), others well here and there only (zones IV, V, VI), whilst others are most capricious, with only scattered cells in samples of several dozen tubules binding the osmium (coni vasculosi, zone I A and B). No reason can be given for these observations. Similar observations by Nassonov (1924) and Parat (1928) on the irregularity of impregnation are amply confirmed. Even in adjacent cells of otherwise satisfactory preparations one may see an impregnation, beautiful in every detail according to prevailing concepts of this organelle, on the one hand, or merely a small amorphous group of osmiophil granules, on the other.

The conspicuous supranuclear vacuolated area in histological preparations is identified with the Golgi area of osmium preparations but not always with a vacuolated area in living cells.

Living cells confirm the length and tenuousness of the stereocilia and of the long filamentous mitochondria in zone I A and B.

Zone I C is a shorter epithelium than I A and is characterized by an especially prominent, supranuclear, clear vacuolated zone, which, toward the end of the zone, becomes less prominent although increasing in volume. The nuclei show regular alignment a few microns from the basement membrane.

The majority of cells in zone I C have terminal processes almost as long as the cell itself and usually projecting at an angle of approximately 45° from the cell surface. Often entrapped in these filaments is an ovoid, homogeneous secretion-globule. The precise relationship of the globule to the filaments could not be resolved. The most favourable preparations suggested that the globule was leaving the cell along a column made by the filaments. The problem may be clarified by electron microscopy. No filamentous continuity between stereocilia and Golgi area as figured by Fuchs was ever seen despite careful search.

The Golgi area is a very conspicuous feature of the living cell in this zone. The area, as suggested by the fixed histological preparations described above, is relatively large, occupying the middle three-fourths of the cell. The area is usually predominantly vacuolated, the vacuoles occurring in chains around the periphery. Occasionally the vacuolation is much reduced so that the area cannot be delimited from the cytoplasm elsewhere. No other correlated cytological conditions seem consistently related to the presence or absence of a Golgi zone.

Space not filled by the nucleus, Golgi apparatus, and mitochondria is occupied by rounded granules. They are similar in form and distribution to granules found in all the other living cells examined.

The nucleus is well defined and rounded, separated from the base of the

ll by only a small quantity of cytoplasm containing the granules referred to mediately above.

The basal cytoplasm, sometimes rounded as in zone I A and B, is squared contour or drawn to a point at one angle of the square as if underlying the unded base of an adjacent cell.

Sections of zone I C stained for mitochondria show filamentous rods filling e apical zone of cytoplasm and ranging down on each side of the Golgi area skirt it and the nucleus, so terminating in the basal portion of the cell as a llection of shorter rods and granules. Most sections suggest that the bulk these filaments form a cylindrical investment to the whole of the cell content, so that when the apical portion of cytoplasm is crowded one may suspect tangential (more peripheral) plane of section. Transverse cuts exemplify this ntention.

Sections of material impregnated with osmium show a very prominent mass osmiophil material immediately above the nucleus, occupying up to 50% the cell volume. Rims of osmiophil material surround chains of vacuoles istributed approximately linearly in the direction of the lumen. There may e three to five of such rows occupying the width of the cell. There is no oubt that this (or its equivalent in other rodents) is the epididymal zone sed in most studies of the Golgi apparatus previously published (Fuchs, 902; Ludford, 1925; Nassonov, 1924; Dalton and Felix, 1954).

There is a close similarity in the appearance of living and fixed cells in this one. The living cells exhibit a better-preserved terminal structure, and the ngth of the stereocilia is much more apparent. Otherwise cytological details f cell size and shape, Golgi area, chondriome, and nuclear size and position re similar.

Zone II. The distinguishing feature is the presence of vacuoles, often of nsiderable size, in the apical $\frac{1}{4}$ to $\frac{1}{3}$ of the cytoplasm. The nuclei are regularly rranged basally. There is no very prominent clear cytoplasmic area as in receding zones of the epididymal duct. Within this zone there is variation to he extent that the more posterior portion has a shorter epithelium with fewer r even no apical vacuoles.

Cells of fresh preparations from zone II, like their fixed and stained counterarts, are identified by the conspicuous vacuolation of the apical cytoplasm. s has been remarked, there is considerable variation down the length of one II in this vacuolation. This is confirmed in the wet preparations. Dissections from the junctional area with zone I C show a shorter, plumper cell ith few apical vacuoles. Samples from the junctional area with zone III milarly show a shorter, plumper cell, whilst in the bulk of the zone are pical tall columnar, highly vacuolated cells. The vacuoles, which all show milar degrees of translucency, are distributed as follows. One constant set immediately supranuclear, arranged in a long U-shaped chain open apically, he centre of the U occupied by more opaque material, also beset with smaller acuoles. This is the classical Golgi area, so conspicuous in this and adjacent nes I C and III; it may or may not be connected directly by a vacuolar

chain with the apical vacuoles which occupy the luminal third of the cell. These vacuoles, two to seven in number and becoming smaller as their number increases, may be found either fenestrating the attachment of a conical terminal secretory mass to the cell, within the apical third of the cytoplasm, or forming a chain down one side of the cell to connect with the Golgi area group.

Mitochondria are prominent in preparations less than one-half-hour old, but after this they either lose their refractility or become granular, so losing their identity amongst the other cytoplasmic components. They are long and filamentous and scattered throughout the cell, sparing the Golgi region and the luminal end of the cytoplasm immediately beneath the border. They may, however, infiltrate between the apical vacuoles.

The stereocilia are shown by these preparations to be three-quarters of the length of the cell, protruding directly out in the direction of the long axis of the cell. Their bases are surrounded with an amorphous mass of low refractility; this is probably secretion, which in most cells is present as a low flat cap over the free surface but occasionally prolonged into a conical or bifid villiform protuberance, the base of which is often vacuolated.

The refractility of the nucleus is low. There is a well-marked nuclear membrane and the nucleus rests against the basal pole with a collection of highly refractile, dark granules between it and the pole of the cell. These granules appear to be continuous with a row of granules of similar refractility lying beside the nucleus and Golgi area, and others scattered in the apical cytoplasm.

Altmann preparations show a distribution of mitochondria similar to that in zone I C, in that rows of smaller rods than those in zone I C are linearly distributed within a cylinder around the periphery of the cell, invading the centre of the cell only at each extremity. Their number appears to be in reciprocal relation with the apical vacuolar content of the cell. The distribution is best confirmed in transverse sections of the cell (fig. 2, c).

Tissues impregnated with osmium confirm the wet preparation picture of the Golgi area. Three or four long chains of vacuoles surrounded by an osmiophil rim occupy the central half of the cell length in a site immediately above the nucleus. The nuclear ends of the chains are more or less defined at one level from cell to cell but the apical end tapers off into the cytoplasm in straggling fashion and the ends of some columns, at least, become continuous with the apical vacuoles. These vacuoles, in turn, are devoid of osmium impregnation (fig. 2, c).

The vacuoles of the Golgi region and those situated apically are seen equally clearly in the living cell, but in the fixed cell only the apical vacuoles are prominent. Otherwise the distribution of the mitochondria, the shape and position of the nucleus, and the appearance of stereocilia is similar in living and fixed cells.

Zone III. The epithelium is much shorter than in the preceding zones and the cells have a supranuclear clear area which occupies most of the cytoplasm

this region. The shape of the nucleus is irregular, varying from ovoid to ore or less rectangular. The margin is often notched or folded. Stereocilia e not prominent in this zone.

Unlike the cells of other zones, zone III cells prepared from fresh bits of sue show cytological detail very clearly. Two cell types appear.

The commoner type is a short, rectangular cell usually without stereocilia ccasionally short, vaguely defined hairs can be seen, about one-quarter of e length of the cell). Homogeneous, slightly refractile material, presumably cretion products, is present in a few cells, where they usually form a low p or flattened cone.

The bulk of the supranuclear portion of the cell is occupied by the Golgi ea, but it is not always as sharply defined from the general cytoplasm as in evious zones because of its content of mitochondria and granules, and because is not so strikingly vacuolated. The general conformity of its vacuoles to spheroid, however, suffices to delimit it quickly in wet preparations.

The nucleus is usually conspicuous and its membrane is clearly notched d often folded. Occasionally a very prominent refractile nucleolus is present.

Characteristically the outline of the basal border of the cell is square rather an round, and contains an area beset with large numbers of small, highly fractile granules. Whilst such grouping and refractility of basal granules is mmon in wet preparations throughout the duct, it appears particularly rikingly in zone III.

The less common cell type is about 10% longer and is narrower, usually ith a more rounded nucleus and a greater content of cytoplasm above the olgi area (fig. 2, F). It is found mixed with the commoner cell in most mples of zone III and on reference to fixed sections, groups of the taller lls are scattered side-by-side with shorter cells.

Preparations stained for mitochondria reveal large numbers of small rods nd granules crowded into the thin rim of cytoplasm surrounding the Golgi ea and even invading this area. These surround the nucleus and are collected gain at the cell base as a clump of granules. The identity of this collection ith the refractile granules of the wet preparations has not been established, ut seems likely. Nassonov's (1924) and Benoit's (1926) observations of the creasing density of the chondriome in this *unter* portion of the organ is rified in the case of zone III.

The negative image of the supranuclear clear zone referred to above and the corresponding area in wet and Altmann preparations is brought into rong relief in osmium preparations as a system of tortuous rods. Vacuolation the rods is present but not as commonly nor as conspicuously as in the evious zones. In this respect the zone simulates the efferent ducts more osely. The Golgi apparatus occupies some 50% of the cell volume, extending om immediately above the nucleus to just beneath the free surface. This lationship holds for both shorter and taller cell types.

The prominence of the Golgi area as a negative image in standard histological eparations and as a positive image in osmium preparations is in marked

contrast to the picture in the living cell, where the Golgi area is not picked out from the remainder of the cytoplasm. However, other features are similar in fresh and fixed preparations, e.g. the lack of prominence of stereocilia, the short mitochondrial rods interspersed with Golgi area, and the basal, irregularly shaped nucleus, whose margin is folded.

Zone IV. This zone is characterized by the presence of prominent juxtanuclear vacuoles, many of which indent the nucleus. The vacuoles may be either supranuclear or perinuclear in extent. The outline of the nucleus is very irregular, owing to marked notching and folding of the nuclear membrane.

Conspicuously vacuolated cells, most of whose cytoplasm is given over to a meshwork of vacuoles, occur very commonly in zone IV. These cells have been designated 'clear cells'.

Wet preparations of zone IV show the juxtanuclear vacuoles to contain an irregular, highly refractile mass occupying about 85% of the volume of the vacuole; the remainder of the content is a clear rim or moat about the mass. An indentation of the nuclear membrane is opposed to the vacuoles, and these preparations show this apposition to be very precise. Chains of vacuoles, often of smaller size than those immediately in association with the nucleus, lead up toward the cell surface and each contains its own irregular refractile mass.

The luminal surface of the cell is lost in a narrow, indistinct, amorphous zone, probably secretion. Stereocilia are not prominent.

The Golgi area is not prominent and is permeated by mitochondria, other granules, and the rows of vacuoles referred to above, rather similar to those of zone III. Vacuolation in the Golgi area itself is not obvious.

In fresh preparations, mitochondria fill the cell throughout its extent except the nucleus. As slender short rods they are often seen disseminated in the Golgi area and concentrated at the cell base.

Indentation of the nucleus reaches extremes in this and the following zones. Notching of irregular depth covers the whole of the surface. The nucleolus is usually prominent.

From its relatively high position in the cell, the nucleus has beneath it a quantity of cytoplasm containing the usual refractile granules and mitochondria.

No recognizable clear cells have been recorded in wet preparations, but within many clumps not broken up by teasing there were certain cells, which although not recognizable when isolated, were presumably cells of this type.

Zone IV B is characterized by the position of the juxtanuclear vacuoles which are both infra- and perinuclear as well as supranuclear. Otherwise the content and relationship to nuclear notching is similar.

The Golgi area and chondriome are similar to those of zone IV A, and the high incidence of binucleation referred to by Reid and Cleland is confirmed. The nucleolus is usually as prominent as in zone IV A.

The mitochondrial arrangement in stained preparations is characteristic. Very numerous short rods crowd all the space in the cell left by the nucleus

l vacuoles, in marked contrast to more anterior zones I and II. Running wn the side of the nucleus in three or four lines they aggregate in greater mbers in the basal cytoplasm (fig. 2, 1). There are large numbers of basal tochondria in these cells. As in the more anterior zones, the more basal tochondria are shorter rods and granules.

The striking feature of osmium preparations of this zone is the marked miophilia of the juxtanuclear vacuoles and of the chains of vacuoles as they oceed toward the cell surface. The Golgi area itself extends the full width the cell and is not markedly rounded as in more anterior zones, the overall ect being the production of a uniform band around the lumen of the tube. common with zones V and VI, vacuolation of the network of osmiophil ds comprising the area is not conspicuous; these rods seem solid through- t all grades of osmium impregnation.

Clear cells are common in this zone and are represented as interlacing reads or small rods surrounding large numbers of vacuoles, giving an overall onge-like appearance. The threads are too tenuous to assess a real colour lue in Altmann-type preparations but they resemble mitochondria. The icleus is often in the apical half of the cell and may be pycnotic. Osmium eparations show substantially the same arrangement of interconnected rods, ch osmiophil, and although the rods are too slender for accurate resolution ere appear to be three or four of them surrounding a nearly spherical space. he impression is, again, that the rods are mitochondria.

The so-called 'vacuoles' of ordinary histological preparations are seen to be most filled in life with a highly refractile mass. For the remainder of cell ructures, e.g. free surface, mitochondria, Golgi apparatus, and nucleus, there close conformity between the appearances of fresh and fixed cells. Thus the olgi area is not prominent in either kind of preparation.

Zone V. In this zone the juxtanuclear vacuoles decrease in number until ey are absent. The nuclei have irregular outlines and are roughly rectangular. he nuclear chromatin, as in the above zones, is distributed in finely granular rm. The infranuclear cytoplasm may be large (zone V A) or very small one V B) in amount.

Clear cells are especially abundant. Binucleate cells are also common.

Living cell preparations from zone V are favourable subjects for phase- ntrast microscopy for the reasons given above. Suspension in serum shows hazy coagulum at the cell surface which is about 10% of the cell height. In otonic sucrose solutions a mass of fine hairs is seen projecting almost rtically from the cell surface. The Golgi zone is vaguely defined in most lls. It is strewn with mitochondria as small rods, and with granules. Occa- onally some cells show a well-defined, rounded, vacuolated area occupying % of the supranuclear cytoplasm. The nucleus is relatively large, notched, nd indented; it contains well-defined nucleoli. This difference in refractive dex between nucleolus and nucleoplasm is a feature of zones V and VI. inucleation is common.

The subnuclear cytoplasm is variable in amount and is characterized by a

content of large numbers of rodlets. No increased translucency or vacuolation of this cytoplasm was seen, such as characterizes zone V A in fixed material.

Juxtanuclear vacuoles of similar form and content to those of zone IV are found in random cells. They are quite distinctive, though not as numerous as in zone IV.

Preparations of postchromed material confirm the density and form of the chondriome as seen in wet preparations, i.e. large numbers of rodlets scattered diffusely throughout the cell, unimpeded by any other organelle.

The Golgi picture is similar to that of zone IV in that there is a tangle of osmiophil rods occupying most of the supranuclear cytoplasm abutting closely on the Golgi area of the neighbouring cell; there is no obvious vacuolation.

There is a general conformity between the appearance of fresh and fixed cells as regards the free surface, cell shape, and nuclear shape. However, the vacuolation of the Golgi area occasionally seen in living cells is not seen in osmicated preparations. Further, the qualitative difference between the cytoplasm above and below the nucleus in zone V A with respect to lightness of staining and appearance of fine vacuoles is not apparent in the living cell. The good definition of nucleoli in living cells of zone V is not a feature of fixed cells.

Zone VI is characterized in histological preparations by the presence of coarse chromatin in the nucleus; it is adherent to the nuclear membrane. Binucleation is common and the cytoplasm is homogeneous in haematoxylin eosin preparations, but contains large granules in sections stained with acid fuchsin. Clear cells are common.

The characteristic feature of the cell picture in wet preparations of zone VI is a variation in shape, which ranges from ovoid-spherical to tall-columnar. In fixed preparations, on the contrary, the epithelium is low and of nearly uniform height. The long axes of each cell must be at varying angles to one another, that of the shortest cells being perpendicular to the basement membrane, that of the longest being very nearly flat or tangential. Careful observation of cell boundaries in fixed material confirms this. Vacuolation is a common feature in the supranuclear region. The cytoplasm is strewn with granules which are larger than mitochondria, have a greater refractility, and appear to be concentrated in the tapering base of these cells. The unusual distribution of chromatin, so typical of fixed preparations, is not a feature of living cells.

The form and distribution of mitochondria in fixed preparations is similar to that in zone V. The granules referred to in the living cells are not obviously identical with fuchsinophil granules common in these cells in Altmann preparations.

The Golgi area, as seen in osmicated preparations, is similar to that in zone V.

The true constitution of the epithelium of zone VI is not revealed by mere inspection of a histological preparation. The variation of cell shape in the wet preparations reveals the true constitution. The nucleus of the living cell fails

show the chromatin distribution so characteristic of sections stained with ematoxylin.

eneral observations on living cells

Apart from the formal description of the structure of living cells, a number general points emerged during the study of numerous preparations.

With attention to minor detail of slide preparation, fresh serum, and tempera- re control, cells showed no observable change in cytological detail beyond e following observation. Up to half-an-hour after mounting (i.e. $\frac{3}{4}$ to 1 h ter death of the animal), cells from most zones are difficult to perceive except a collection of spherical granules interlaced with mitochondria. This is rticularly true of zone IV A and B. The cells resemble a conglomerate of heres of varying size, with no cell or nuclear outline visible. After this time, ite insidiously, the cell outline materializes, the nuclear membrane becomes stinct, and fine hairs, the stereocilia, are seen. No illustrative photographs e available, since the changes are below the resolution limits of the photo- icrographic apparatus used. Comparison of exposures before and after this hange fail to bring out its subtlety. After 48 h (and sometimes as early as 2 h in zone II), clear vesicles appear from random points on the cell surface, aching up to 50 μ in diameter after a few hours, evidently by coalescence, nd distorting the cell in bizarre fashion. Short of this gross disruption there no evidence from close scrutiny that the cell detail is altered in any way om that in well-fixed material.

Apart from the ciliary movement found in efferent duct cells there is no preciable movement in other cell components. Thus stereociliary hairs, acuoles in the Golgi area and those near by in the apical cytoplasm (zone II), e secretion cupolas at the cell surface, and the nuclear membrane were nchanged over prolonged periods of observation. Nothing equivalent to the ovement of pancreatic zymogen granules (Hirsch) or to currents, as in ant cells, which might account for the transport of particles of pigment Mason and Shaver, 1954), was to be found.

Preparation of suitable disaggregated cell suspensions varies with the zone. oth efferent ducts and epididymal duct down to the end of zone II are fficult to fractionate in this respect. In contrast, more caudal zones produce rapid yield of free cells. In any case, merely allowing the tissue to remain at 5° with or without added homologous serum, provided steps are taken to void dehydration, will produce free cells after an interval of about 2 h. here is no resolvable difference in cells prepared thus compared with those mpled immediately after death, and left under the microscope for an equal me. In other words, cells left for some time before teasing have apparently ndergone the small phase-change referred to above, whether they were in e organ or on the slide. The enzyme hyaluronidase was used in an attempt facilitate disaggregation, without appreciable effect.

The value of varying the refractive index of the suspending agent was orne out when resolution of fine structure like stereocilia was required. When

the stereocilia were very short or tenuous, more watery media (isotonic sucrose) gave much better visibility.

Discussion

Integration of these findings with those of earlier workers has been made difficult by the inability to localize the zones to which they referred by reference to the gross anatomy of the ducts. However, the non-motility of the stereocilia is amply confirmed and the much figured Golgi area of zone I C and II of the studies of Aigner (1900), Benoit (1926), Nassonov (1924), Ludford (1925), and Dalton and Felix (1954) is placed in its correct sequence down the length of the tube. The distribution of mitochondria supports the findings of Nassonov, Ludford, and Benoit as regards increasing density and decreasing size toward the tail, and absence from the Golgi area in cranial zones. These authors have generally stressed the basal concentration of mitochondria, which is amply confirmed here. Besides mitochondria, wet preparations show that granules, although occurring ubiquitously in the cytoplasm of all cells studied, are also concentrated basally. There was no relationship between these most obvious basally situated granules and any cell component of fixed preparations stained for mitochondria, except in zone VI. Benoit raised the question of association with ergastoplasm in this connexion; alternatively it may be associated with some cell activity in relation to the basement membrane, e.g. secretion.

The problem of the exact nature of the free cell surface in the epididymis is still in doubt. Homologous serum has disadvantages, as has been pointed out, but even with more watery fluids it is difficult to resolve any organized structure in the end of the efferent duct cell, although postchroming methods do reveal a brush border on some cells. This accords with Benoit's observations. He draws attention to the similarity of such terminal processes to those in other secretory cells, e.g. the gut and placenta. The classical stereocilia of zones I and II appear in these studies to be no more connected with cell activity than that they occasionally enmesh a secretion globule. This agrees with the description of Fuchs on this point, although observation failed to reveal any intracytoplasmic connexion with the Golgi zone as figured by this author. His preparations, however, were of the mouse, where they may be more obvious, and he used iron haematoxylin staining. Likewise Laurent (1932) traced the course of the hair roots to the supranuclear region in the epididymis of the guinea-pig, but gives no illustrations. This has not been found in the rat. The main feature brought out by the examination of living cells in this study is the extraordinary length of these hairs, and the ease with which they are damaged mechanically. Where the cell is disaggregated with a minimum of teasing, e.g. zone III, long stereocilia are regularly seen. These hairs appear to be rigid throughout their length, which would weigh against the suggestion that they might be a mechanism for increasing the secreting surface, after the style of an overgrown brush border.

The position of apocrine secretory knobs is confirmed in efferent ducts and

zone I, occasionally in zone II. Benoit thought they did not exist in the living ll. Whilst this would be very difficult to investigate, having regard to the acity of the most tenuous efferent ducts, it does not seem likely that gross d rapid changes in shape follow the death of the animal and the subsequent olation of the cell.

The findings here presented bear at some points on the enigma of the Golgi mplex. Where it is represented in what has been called classical form ones I and II), the structure of the complex in living cells accords closely ith the detailed account given by Baker (1945, 1957). A substantial proporon of cell area near the nucleus is given over to chains of vacuoles in heroidal mass surrounding a central, less vacuolated area. The osmicated aterial accords point-for-point with this interpretation and the age-old conntion of artifacts in such impregnation is no longer significant. However, in nes III, IV, V, and VI, where there is a more or less well-defined vacuolated ea in living cells (although not as clear cut as in the classical zones), the rresponding osmicated areas are not vacuolated, but more in accord with e usual description of a network of osmiophil rods or plates (the 'classical etazoan' structure of Benoit). Nassonov (1924) suggests that the degree of mpregnation determines the appearance of vacuoles. Whilst this may be true f zones I and II (his plates seem to correspond with zone II), even the lightest inding of osmium in more caudal zones fails to reveal chains of vacuoles. enoit also observed the absence of vacuoles in the Golgi of the tail region, escribing it as a network of black trusses with irregular contours. No such ounterpart could be found in the living cells and one must conclude that in ese specific zones the Golgi complex *is* indeed altered by osmium impregnaon. In summary, the concordance of findings in living and osmicated material dependent on the zone under consideration. This, of course, does not lucidate how the osmium alters the vacuolated area in the zones concerned. t is of interest here to correlate this with the other clear-cut distinction etween the two groups of zones in question; that is to say, the intimate ssociation of the chondriome with the Golgi area in the caudal groups. This one major factor in the lack of precise definition of the Golgi picture. alton and Felix (1953) refer to and picture the Golgi network, but obviously nly one optical section can be photographed and their illustrations conform ore nearly to the concept of a system of rods associated with vacuoles in a heroidal mass than to a network. Such condensed positive images were rarely en in the cells that formed the subject of this study.

The continuity of the vacuoles of the Golgi complex with those of the ical cytoplasm in zone II was seen many times in fresh and fixed material nd the original observations of Fuchs in this respect were amply confirmed. he association is more vivid in the fresh cells, where the vacuoles of both arts show equally clearly, than in routine stained sections, where the vacuoles f the Golgi area take up more of the counterstain. Elsewhere in the epididymis e association of the Golgi area with cell function is not obvious from these udies. Parat and Nassonov remarked on the variability of the effects of

osmication. One should avoid drawing conclusions too confidently from the study of osmicated preparations. A recent paper by Baker (1957), pointing out the variety of chemical combinations into which osmium tetroxide can enter and the subsequent physical union that it can make with non-specific substances, is interesting as a background to the possible causes of the variety of effects mentioned by Parat and Nassonov.

The picture presented here of the mitochondria is substantially in agreement with that obtained in earlier studies. Their transient appearance as rods in living cells may be simply due to the violence of handling during teasing. The interesting point is that the time of disappearance varies in random fashion from $\frac{1}{2}$ to 4 h and a traumatic cause is more likely than some deficiency in the medium. The mitochondria were the most labile of the cell components studied.

The association of mitochondria with the Golgi area in caudal zones may be significant from the viewpoint of the proponents of the direct transition theory. On the other hand, they may represent a granular secretion product of the Golgi apparatus in those zones, as initially proposed by Fuchs and supported by Nassonov.

The basal concentration of mitochondria may be of significance in connexion with a suggestion of Benoit that in the epididymis of the horse, nuclear material in the form of granules remains in the basal cytoplasm, awaiting transfer across the basement membrane to near-by wandering tissue cells and so to capillaries.

The lack of appreciation of the sequence of zones has led, in the past, to a good deal of confusion as regards the significance of the juxtanuclear vacuoles of zone IV. They do not seem to have been separated from the Golgi apparatus either morphologically or physiologically by Benoit or Ludford. The latter author uses the term 'complex granules' for these vacuoles after Nassonov, who, in turn, described a large granule surrounded by a fluid space in cells of the seminal vesicle of the mouse. There is no doubt from Nassonov's description that he refers to a similar structure as the contents of the juxtanuclear vacuoles of zone IV: 'Jeder Granula stellt ein stark glänzendes gelbliches Körnchen vor, welches von einem hellen Rande von Wahrscheinlich flüssigerer Konsistenz umgeben ist.' Moreover, from his plates the vacuoles do appear to be intimately associated with the Golgi apparatus. However, in the present study, an entire sequence of stages from intimate contact with the nuclear membrane to the typical row of vacuoles along one side of the cell in both vital and fixed material show that these extraordinary envacuolated granules are apparently more concerned with the nucleus than with the Golgi apparatus. The other proposition, put forward first by Benoit in 1921 for the bull, confirmed by Ludford for the mouse, but subsequently refuted by Benoit in 1926, was that these vacuoles may enclose nucleolar fragments extruded by the nucleus. The close apposition between such vacuoles and an indentation of the nuclear membrane would easily account for such a false impression from all but the most careful observation in living

lls. This is Benoit's explanation of his earlier misapprehension and is borne t in these studies. Christie (1955) recognized them as closely related to the ucleus. He called them juxtanuclear bodies and showed histochemically that ey consisted of phospholipid and cerebroside, the phospholipid being dis-ibuted peripherally as a thin rim; this may well correspond to the fluid ace of vital preparations. Baker (1957) draws attention to the presence of milarly constituted 'globules' in the sympathetic ganglion of the rabbit. In esaging the ubiquitousness of these globules in other cells Baker suggests e name *cerephos* globules.

The status of the 'clear' cells of zones III and IV is not clarified by these udies. The findings are in agreement with Nassonov as regards the de-eneracy of the Golgi area, although there is no evidence of his concept of its xplosive rupture and dissemination throughout the cell. Nuclei are not ways pycnotic and the cell often appears to be formed by the confluence of hree or four cells. The failure to recognize these cells in living preparations is nteresting and may mean that they are unusually fragile and do not survive easing.

Polymorphism of nuclei in zones III and IV, first studied by Regaud, is lly confirmed. Most elegant pictures of nuclear pattern are seen in living ells. However, there is no evidence from this study that the deep fissures, hich almost transect the nucleus, proceed to nuclear division, as first postu-ated by Regaud and supported by Benoit. Although their frequencies vary, here are binucleate cells in all parts of the epididymis, unrelated to nuclear olding. The problem must await quantitative studies, for short of actually eeing the two portions move apart, the contact between them is extremely ntimate and the plane of transection would not allow the two separate parts o be seen until they were well separated. By that time, of course, a normal itosis would have effected the same result.

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FIG. 1 (plate). A, efferent duct of the terminal zone. 3-μ paraffin section of material fixed in Helly, postchromed 2 days at 37°; stained by toluidin blue, aurantia. The mitochondria in the apical half are filaments, clustered together subapically, whilst basally they are shorter rods. Conspicuous granules surmount the nucleus in cone-like masses.

B, efferent duct of the initial zone. 3-μ paraffin section of material fixed in Helly and postchromed 2 days at 37°; stained by toluidin blue, aurantia. The apical mitochondria are filamentous, the basal are shorter rods.

C, efferent duct of the initial zone. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. Two well-defined ciliated and several principal cells are seen. The osmiophil material in the former is distributed as a supranuclear cap. This is continued into the cell base as a mass of filaments and granules. In the principal cells, the osmiophil material surmounts an unusually clear area of cytoplasm at some distance from the nucleus.

D, efferent duct of terminal zone. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The osmiophil strands, representing the Golgi area, are situated much closer to the nucleus.

E, zone I A. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The Golgi area is immediately supranuclear and osmiophobe vacuoles may be seen in it. The area is quite small compared to the length of the cell. The nucleus occurring between the middle and right-hand cell borders is that of a 'halo' cell.

FIG. 2 (plate). A, zone II. 3-μ paraffin section fixed in Helly, postchromed 2 days at 37°; stained by toluidin blue, aurantia. The mitochondria are short rods, peripherally distributed. The apical vacuoles characterize this zone histologically.

B, zone II. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The Golgi area is extensive and composed of 3 or 4 rows of osmiophobe vacuoles surrounded by osmiophil rims. The sharp contrast between osmiophilia of the Golgi and the apical cytoplasmic vacuoles is evident.

C, zone II. 3-μ paraffin transverse section of material fixed in Helly, postchromed 2 days at 37°; stained by toluidin blue, aurantia. The peripheral distribution of the chondriome is shown. The central vacuolated area is almost devoid of mitochondria.

D, zone IV, living cell from teased preparation suspended in homologous serum and photographed under phase-contrast microscopy. The nucleus is just below the middle of the cell and is surrounded by four cerephos granules in varying planes of focus. The granules are typically related to notches in the nucleus. The clear moat surrounding these granules is best seen in the lower right-hand granule.

E, zone III. Living cell from teased material suspended in homologous serum at 37° and photographed under phase-contrast microscopy. The apical border is devoid of secretion. The Golgi area is ill-defined because of its content of refractile granules. Numerous granules can also be seen at the cell base. The nucleus contains a prominent nucleolus.

F, zone III. Living cell from teased preparation suspended in homologous serum at 37° and photographed under phase-contrast microscopy to show the alternate cell shape in this zone, which is not so manifest in fixed preparations. The nucleus is rounded and is just outside the plane of focus. It is surrounded by granules.

G, zone III. 3-μ paraffin section fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The photomicrograph shows the relatively large size of the Golgi area, corresponding to the 'clear' area of haematoxylin / eosin preparations and the absence of vacuoles within the osmiophil rods.

H, zone III. 3-μ paraffin section fixed in Helly, postchromed 2 days at 37°; stained by the Bensley–Cowdry modification of Altmann's aniline fuchsin. The mitochondria are short plump rods crowded about the periphery of the cell, but also invading the Golgi area.

I, zone IV. 3-μ paraffin section fixed in Helly, postchromed 2 days at 37°; stained by the Bensley Cowdry modification of Altmann's aniline fuchsin. The mitochondria are short rods scattered throughout the cytoplasm, about the periphery of the nucleus, and in the subnuclear cytoplasm. The prominent fuchsinophil nucleolus is often seen in zone IV. The nuclear membrane is notched.

FIG. 3 (plate). A, zone IV. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The Golgi material forms a uniform band around the tubule and is non-vacuolated. The cerephos granules are markedly osmiophil and their apposition to the nucleus is again apparent.

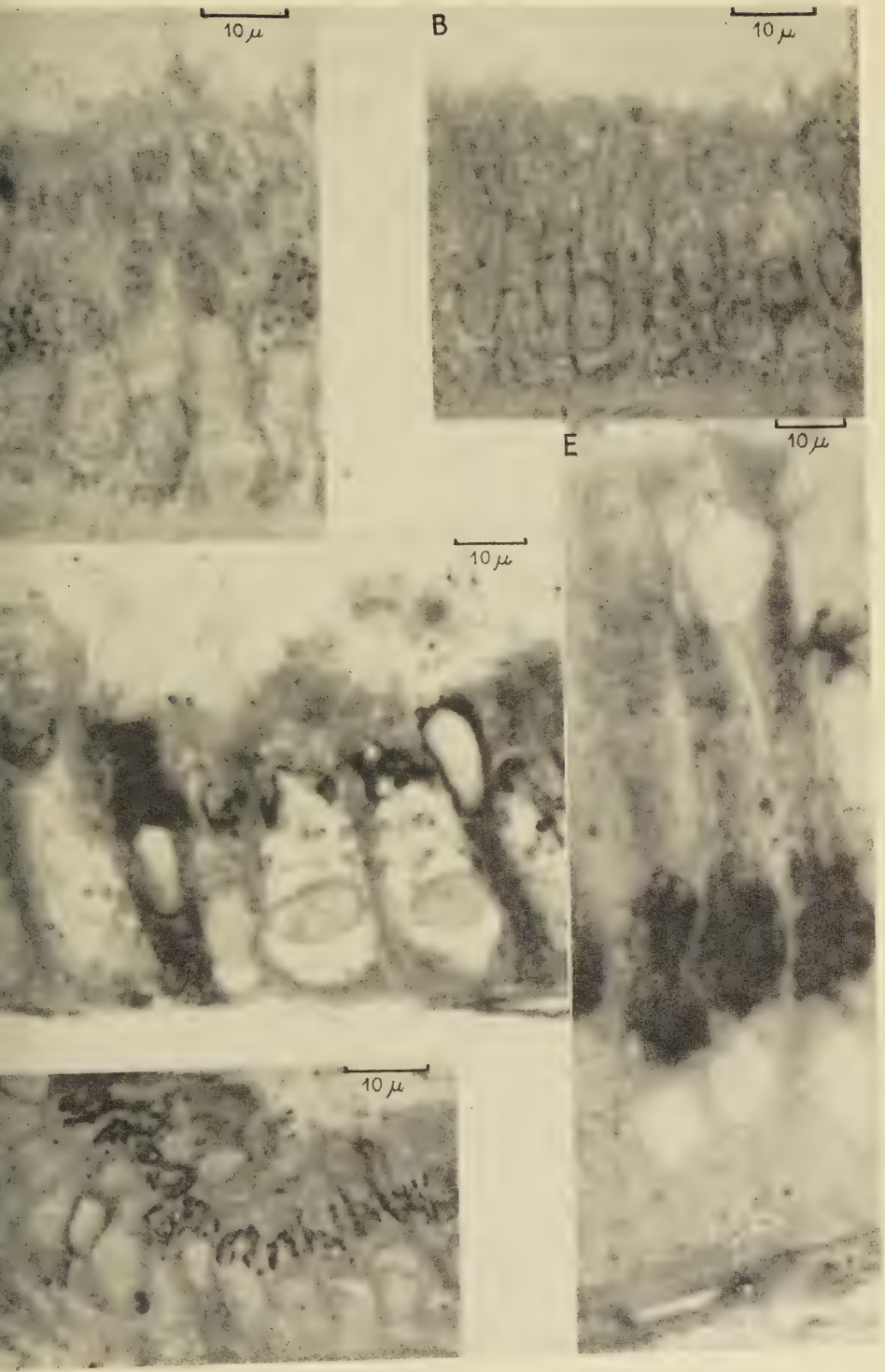

FIG. 1
B. L. REID

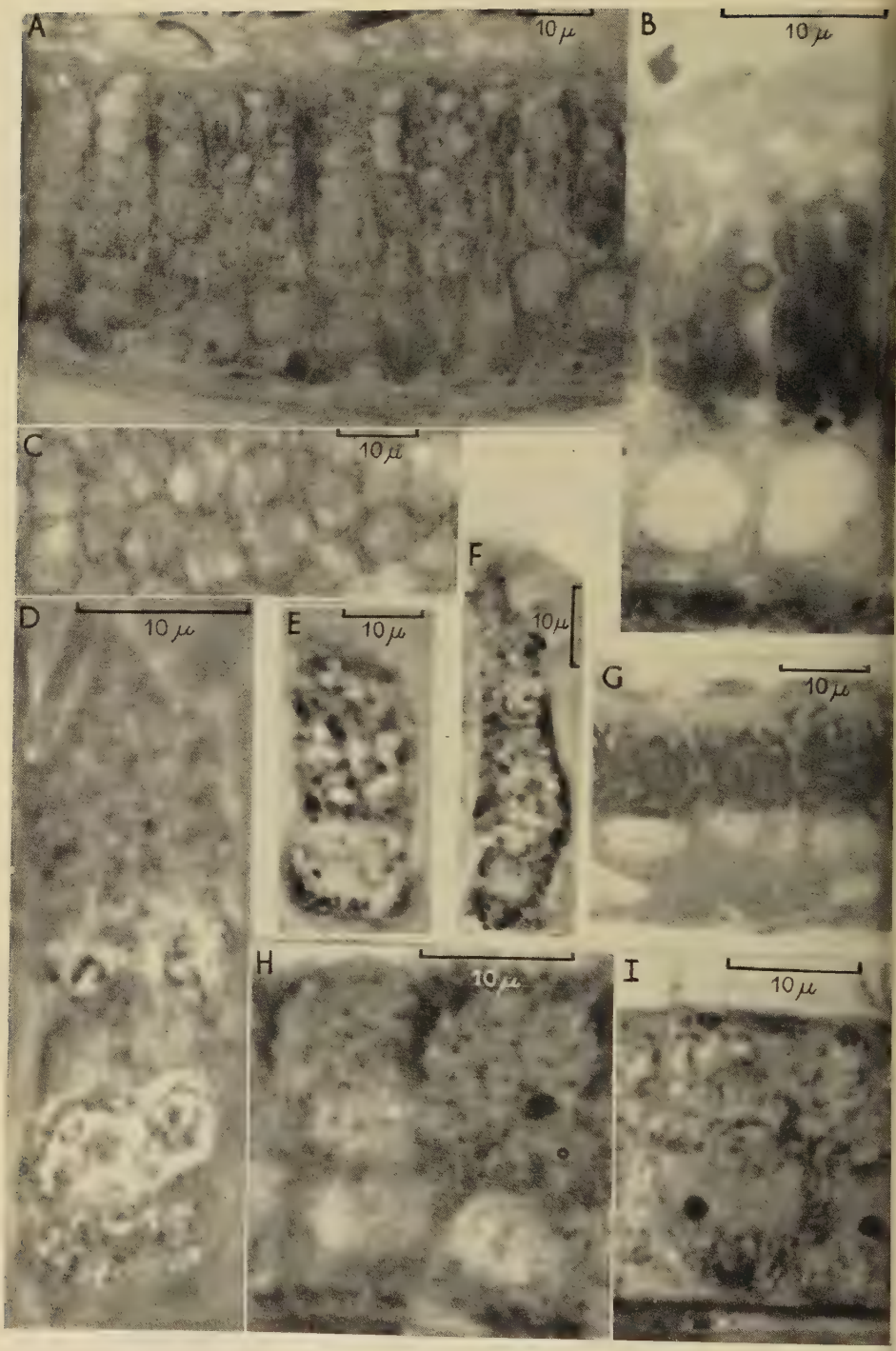

FIG. 2
B. L. REID

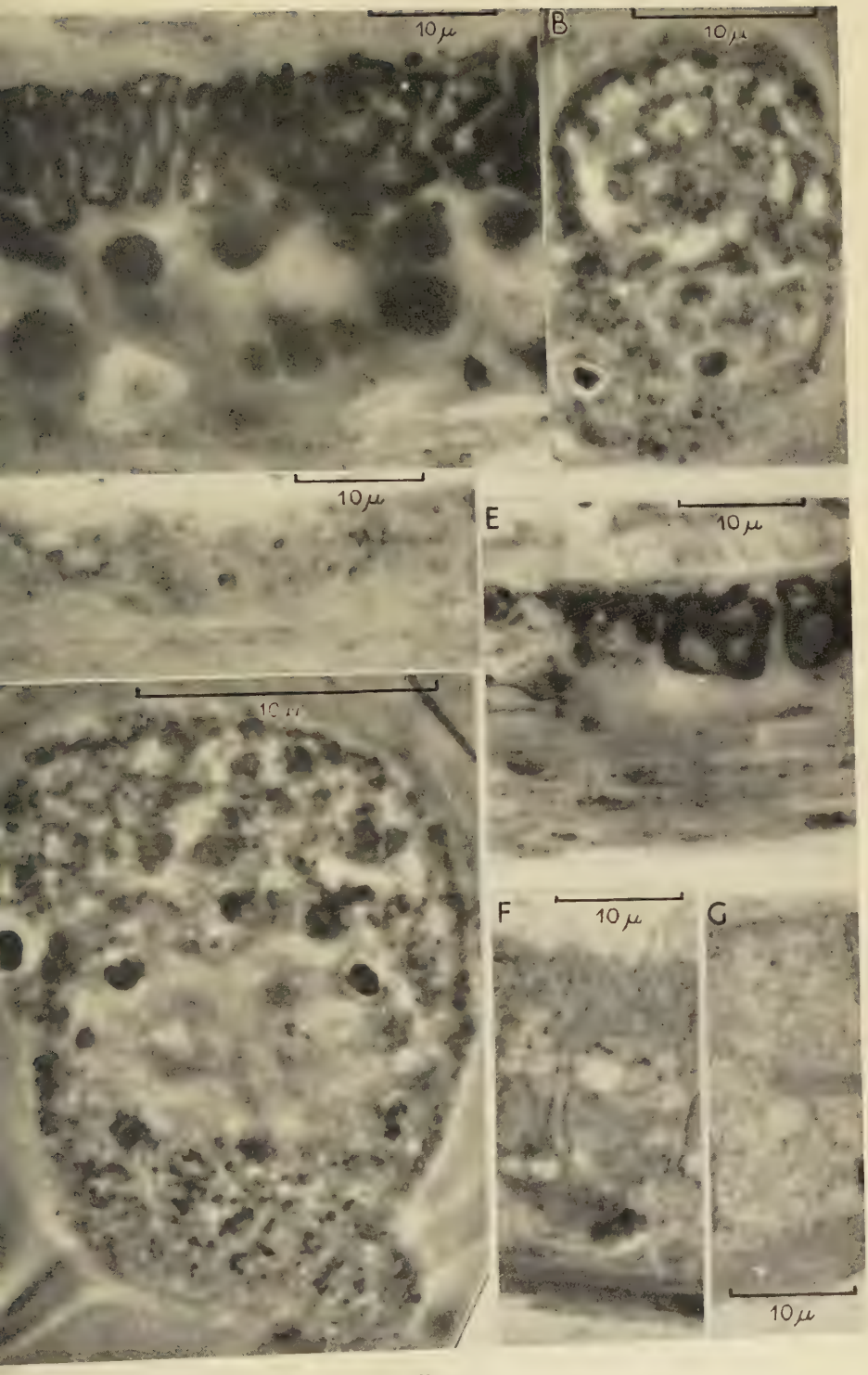

FIG. 3
B. L. REID

B, zone V. Living cell from teased preparation suspended in homologous serum and photographed under phase-contrast microscopy. The Golgi area is vaguely demarcated by a ring of vacuoles occupying most of the supranuclear cytoplasm. The nucleus is ovoid and is indented in the transverse axis of the cell. Small granules are concentrated at the cell base. The cerephos granule is present at the left-hand side of the basal cytoplasm.

C, zone VI. 3-μ paraffin section of material fixed in Helly, postchromed 2 days at 37°; stained by toluidin blue, aurantia. The epithelium is very short and the lower half of the picture is taken up with the muscle-coat, which is thick in this zone, as the vas deferens is approached. About 5 epithelial cells are shown. The mitochondria are inconspicuous short rods scattered throughout the cells and the muscle-coat. The larger granules, more apparent toward the centre of the picture, are apparently separate from mitochondria and may represent some sort of secretion product.

D, zone V. Living cell from teased preparation suspended in homologous serum. At a higher magnification than fig. 3, B, to show small granules present throughout the cytoplasm, including the Golgi area. The latter is not defined. The nucleus is ovoid and has three cerephos granules associated, the upper right-hand one of which is seen in characteristic close apposition with the nuclear membrane.

E, zone VI. 3-μ paraffin section of material fixed in Mann's fluid; Ludford's modification of Mann's method of osmication. The Golgi material is in the form of black trusses which are not vacuolated. The interruption to the continuity of the Golgi material as a band around the nucleus, in contrast to fig. 3, A, is apparently the result of the heterogeneous constitution of the epithelium in this zone.

F, zone IV. 3-μ paraffin section of material fixed in Helly, postchromed 2 days at 37°; stained with toluidin blue, aurantia. The widespread distribution of the small rod-like mitochondria is shown. The perinuclear vacuoles are the sites of the cerephos granules.

G, zone IV. Same technique as F. Clear cell containing large numbers of small spaces surrounded by small rods which stain like mitochondria.

Histochemical and Morphological Studies of the Lipids in Oogenesis. I. *Periplaneta americana*

By VISHWA NATH, BRIJ L. GUPTA, AND BACHAN LAL

(*From the Department of Zoology, Panjab University, Hoshiarpur, Punjab, India*)

With one plate (fig. 6)

Summary

. Three kinds of lipid bodies have been described in the oogenesis of the cockroach, *iplaneta americana*: (i) L_1 bodies, present in the earliest oocyte, which persist till oocyte measures approximately 0·5 mm and contain phospholipids only, possibly ing more lecithins than cephalins; (ii) L_2 bodies, which first arise in the oocyte asuring 0·4 mm and have a complete or incomplete sheath of phospholipids surnding a medulla of triglycerides (rather highly saturated); (iii) L_3 bodies, which the only type of lipids present in the oocytes measuring more than 0·65 mm and sist of triglycerides only (rather highly saturated).

2. Some of the larger L_3 bodies give a 'ringed' or 'crescentic' appearance in Sudan ck when used at room temperatures (12° C to 40° C) but appear mostly solid when s colouring agent is used at 60° C.

3. Mitochondria, which remain as fine granules throughout the course of oogenesis, ntain proteins and phospholipids. They seem to have some lipids which are masked rmally but are unmasked after acetone extraction, with a resulting increase in danophilia.

4. Yolk globules appear in the oocytes measuring approximately 0·5 mm. They ntain a protein-carbohydrate complex.

5. The bacterioid objects described by earlier workers have been shown to contain ospholipids and free fatty acids. They possibly play an active role in the lipid nthesis of the cell.

Introduction

NATH and Mohan (1929) studied the egg of *Periplaneta americana* by the techniques of Kolatchev, Mann-Kopsch, Champy-Kull, Da Fano, d Bouin-iron-haematoxylin, and by staining fresh coverslip preparations th neutral red, or osmicating them in 2% osmium tetroxide. They arrived the following conclusions, which were subsequently fully confirmed by resson (1931).

The 'Golgi elements' can occasionally be seen in the young oocytes without e aid of any vital dye. Neutral red stains them weakly. They darken ghtly after short periods of immersion in 2% osmium tetroxide. In form e so-called Golgi elements are described as hollow vesicular bodies with a stinct osmiophil rim and a central osmiophobe substance. With the growth the oocyte a large number of these bodies grow enormously, store up fat in eir interior, and give rise to the fatty yolk. In this process of enlargement e rim of the so-called Golgi vesicles becomes more and more attenuated. nese authors also describe mitochondrial granules, and nucleolar extrusions nich form the so-called albuminous yolk.

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In the present investigation a large number of modern histochemical tech niques have been employed to work out the chemistry of the so-called Gol bodies, mitochondria, and yolk. The most important conclusions are (1) tha at no stage in the course of oogenesis do the so-called Golgi bodies appear i the form of an 'apparato reticolare' as originally described by Golgi (1898 and (2) that the so-called Golgi bodies are in reality lipid spheres of at lea three types.

Material and Technique

Specimens of *P. americana* were collected locally from Hoshiarpur. Onl sexually mature specimens were used. The material was available all the yea round except during the months of severe winter. It was found that the cyto plasmic contents of the oocytes showed considerable variations in their size amount, and chemical composition according to the season in which th material was fixed.

The animals were dissected alive in a wax dish. On opening the body cavity the fixative to be used was poured into it to minimize the post-mortem changes. The ovarioles were then removed along with their terminal filament by means of fine forceps and transferred to the fresh fixative in a glass stoppered capsule. The details of the various fixatives employed, the em bedding media used, as well as the various staining techniques tried, hav been summarized in table 1 on page 328.

In addition to the routine methods, various lipid extractions were als tried. The solvents used were cold acetone, cold ethanol, cold ether, col methanol plus chloroform, cold methanol plus ether, hot acetone, hot ethano and hot ether. The last three were used in a Soxhlet extractor. The followin three distinct methods have been employed for these extractions:

(1) Fresh ovarioles were treated directly for 24 h with each of the abov solvents, the material was brought to water quickly through the descendin grades of ethanol and fixed in formaldehyde calcium (Baker, 1946) for 6 h postchromed as usual, and embedded in gelatine.

(2) The ovarioles were first fixed in formaldehyde calcium for 6 h an embedded in gelatine without postchroming; sections were cut at 10 μ. Thes sections were subsequently treated for 24 h with each of the above solvent and coloured either with Sudan black B or acid-haematein test of Baker (1946

(3) The gelatine sections of the material fixed by the formaldehyde calciu postchroming technique were also treated with all the above solvents an coloured with Sudan black B, Nile blue (Cain, 1947), or acid-haematei (Baker, 1946).

Results

General structure of the ovariole

The ovaries of *P. americana* contain 8 ovarioles each, which are panoisti that is, there are no definite nurse-cells. The ovariole begins as a thin threa containing nuclei and lipid granules, but the cell boundaries are not discernibl in this region (fig. 1, A). It continues as a region in which the cells, althoug

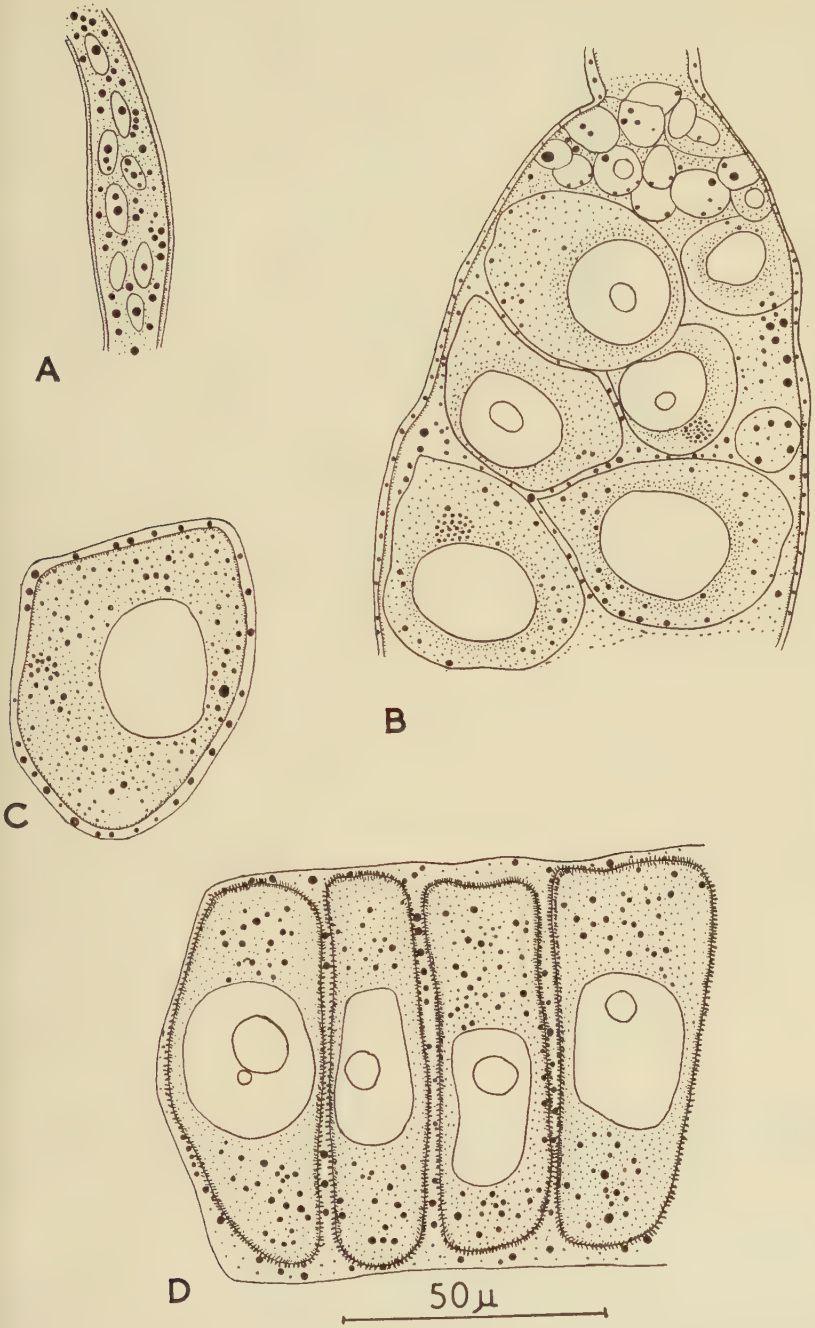


FIG. 1. Camera lucida diagrams drawn from gelatine sections of formaldehyde calcium material, postchromed; coloured with Sudan black B. The distribution of mitochondria, lipid bodies (L_1), and 'bacterioid forms' is shown. A, anterior end of germarium; B, distal end of germarium; C, a very young oocyte from the vitellarium; D, a portion of the anterior region of the vitellarium.

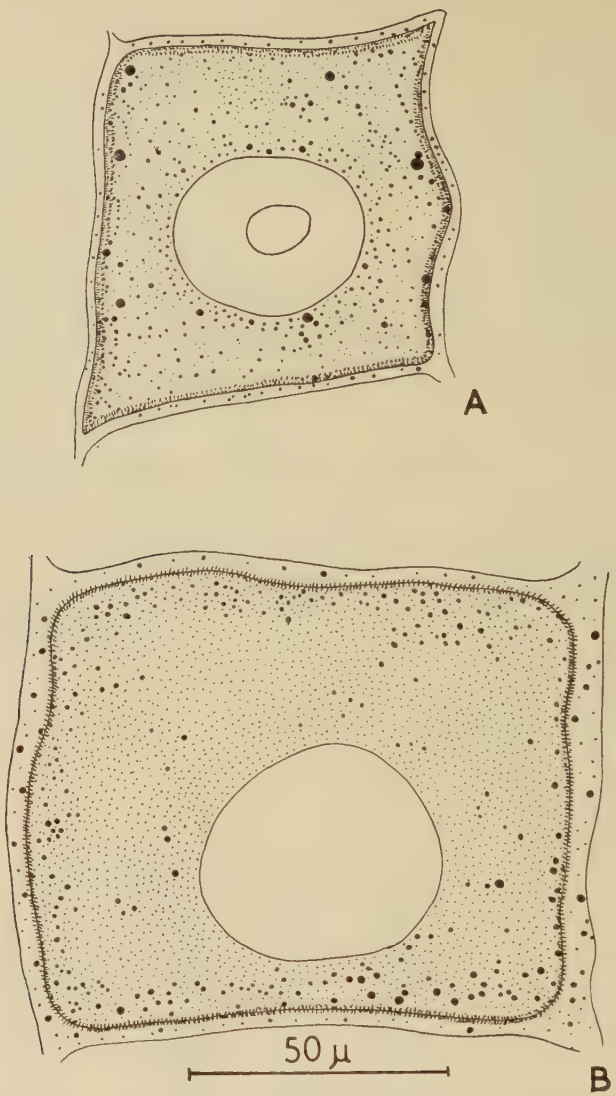
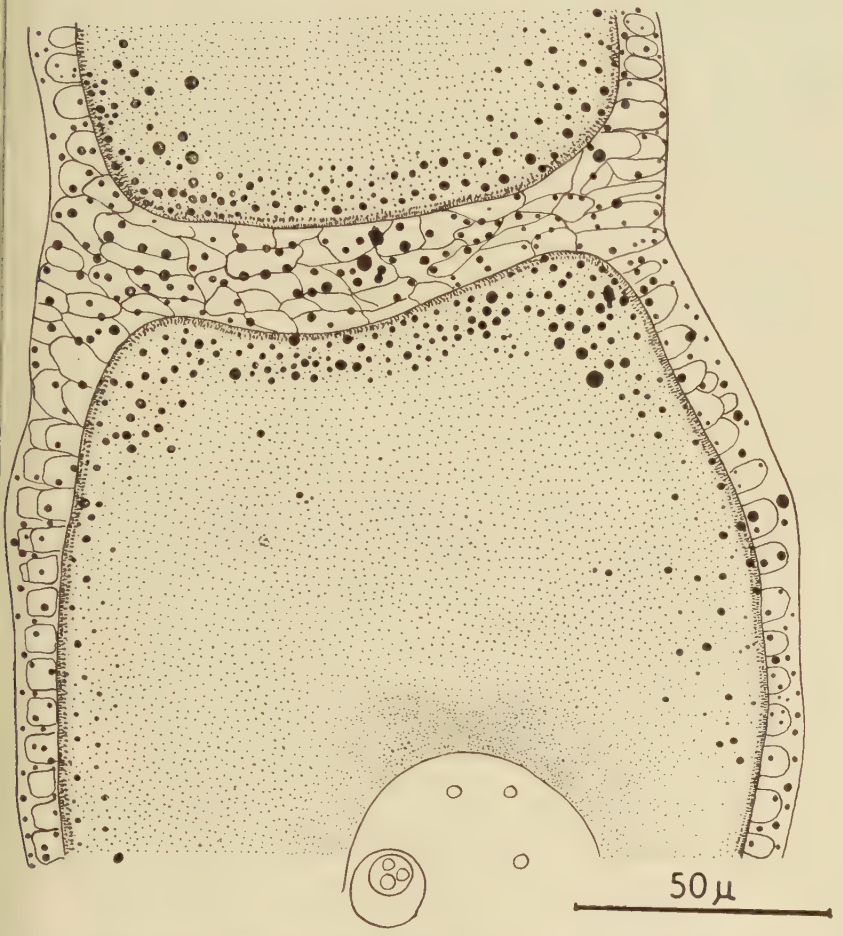


FIG. 2. Camera lucida diagrams from gelatine sections of formaldehyde calcium material postchromed; coloured with Sudan black B. The drawings show the growth and distributio of the mitochondria, lipid bodies, and 'bacterioid forms' in the growing oocytes. A, oocyt measuring 0·075 mm; B, oocyte measuring 0·095 mm.

arranged in two or three rows, show distinct cell boundaries (fig. 1, B). Bot these regions constitute the germarium. This is followed by a long vitellarium It has been noticed that the vitellarium generally terminates in two larg 'yolky' oocytes throughout the year except in March and April, when th number of these terminal 'yolky' oocytes is three.

The present investigation reveals that the lipid contents of the oocyte

dually increase till the oocyte attains a length of approximately 0·1 mm, en the lipid bodies migrate towards the follicular epithelium and show a line both in size of the individual granules and in number (figs. 1, A–D, and A, B). When the oocyte attains a size of approximately 0·2 mm, the number d size of the lipid bodies start increasing, and they start migrating once



. 3. Camera lucida diagram from gelatine section of formaldehyde calcium material, tchromed, coloured with Sudan black B. A part of two adjoining oocytes and the inter-icular region are shown. The lipid bodies (L_1) are mainly concentrated at the ends of the oocytes (size of oocyte approximately 0·28 mm).

ain towards the centre of the oocyte (fig. 3). The increase in the amount of id granules continues without any detectable change in their chemical com-sition till the oocyte attains a size of approximately 0·4 mm (fig. 4, A). uring these stages the larger lipid spheres are generally concentrated towards periphery of the oocyte. Their distribution at the periphery of the oocyte o becomes more uniform (fig. 4, B) as compared with the earlier stages of

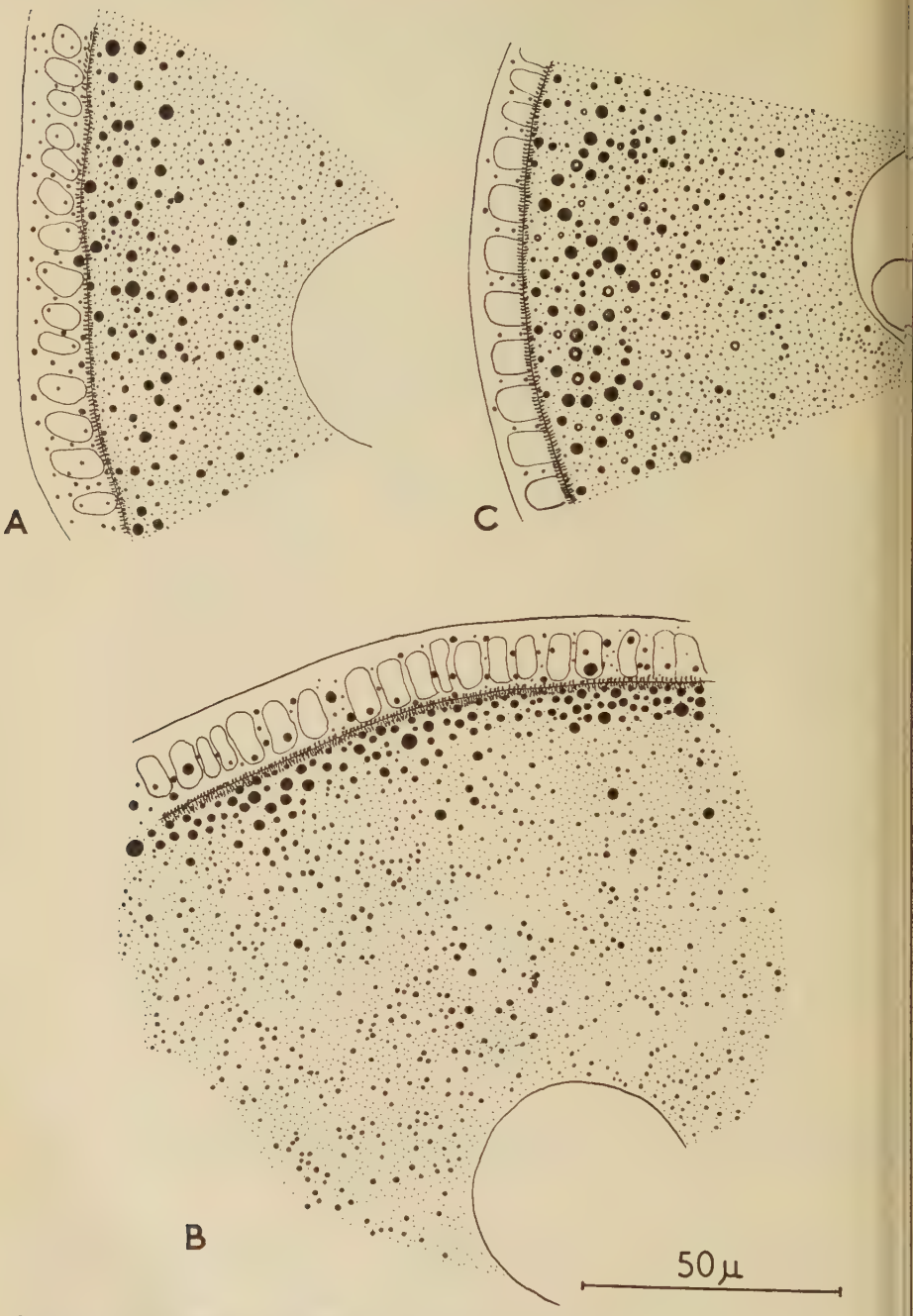


FIG. 4. A and B, portions of oocytes from gelatine sections coloured with Sudan black. The drawing shows the homogeneous nature of the L_1 and L_2 bodies. C, a portion of the oocyte from an acid-haematein preparation. The drawing shows the duplex nature of the L_2 bodies. Unchanged L_1 bodies are homogeneous (size of oocyte: A, 0·42 mm; B, 0·36 mm; C, 0·45 mm).

wth, when they are mainly concentrated near the interfollicular regions of oocyte.

The lipid bodies of the oocytes continue to increase in number. It appears t lipid synthesis occurs mainly near the follicular epithelial cells and the w bodies continue to migrate towards the centre. The oocyte measuring mm or more shows the concentration of the lipid bodies in the central ion, the periphery being occupied by the growing yolk globules (albumi-us yolk). Some of the lipid bodies now attain a considerable size.

The oocytes measuring approximately 0·6 mm generally show the first signs yolk bodies (albuminous yolk), which appears in the form of minute nules in epithelial cells as well as in the cytoplasm adjacent to the epithelial er. These show a quick increase in number and size of the individual dies. Ultimately the larger yolk spheres come to occupy the whole of the toplasm of the oocyte. Lipid bodies in these stages become sandwiched tween the yolk globules.

The follicular epithelium is always one cell thick and is the only source of pply of the raw material to the developing oocyte. In the interfollicular ion, however, the epithelium is many cells thick (fig. 3). The cytoplasm the follicular epithelial cells always contains large quantities of lipid bodies various sizes.

It must be mentioned here that an ovariole always reveals a fairly thick er of small rodlets at the extreme periphery of the cytoplasm and immedi-ly below the follicular epithelium (figs. 1, D; 2; 3; 4, A, B). These rodlets e sudanophil except in very late oocytes, where they remain uncoloured by ost of the techniques employed. They correspond to the 'bacteria' described the earlier authors (Blochmann, 1884, 1887; Nath and Mohan, 1929). nce they appear to contain large amounts of free fatty acids in early stages vitellogenesis (see below) and since they are sudanophil up to quite a late ge, they seem to be concerned with the lipid synthesis of the oocytes.

stochemical observations

The various histochemical tests tried and their results have been tabulated page 328.

Mitochondria. The mitochondria, which are always in the form of minute anules, are present in the cytoplasm of all stages of oogenesis, from the rmarium to the oldest oocyte in the vitellarium. Their distribution in the toplasm is also more or less uniform except in very young oocytes, where me of them are arranged in the form of a ring round the nucleus. Their size d chemical composition remain unchanged throughout oogenesis.

The various histochemical tests listed in table 1 on page 328 show that the tochondria in this material are rich in phospholipids and proteins (AH+, C– and Hg–BPB+); they do not contain any triglycerides or carbohydrates. ey do not seem to play any part in the process of vitellogenesis.

When the ovarioles are extracted with cold acetone or boiling acetone,

either fresh or after short fixation in formaldehyde calcium, the intensity o colour of the mitochondria with Sudan black B either remains unchanged o in some cases is actually enhanced. This is especially the case when fresh ovarioles are extracted with the solvents. The increase in the intensity o colour is also noticeable even when an ethanol solution of Sudan black B i employed. This increase in colour might be attributed to unmasking by the action of solvents, as described by Lovern (1955); that is, to the release o certain protein-bound phospholipids.

Lipid bodies. The various histochemical tests show that the lipid bodies in this material can be divided into three categories, L_1, L_2, and L_3.

The lipid bodies of the first category, L_1, are present in oocytes of diameters up to approximately 0·4 mm; these give a uniform histochemical reaction irrespective of the size of the individual granules, which varies from the granules only slightly larger than the mitochondria to 5 μ (figs. 1–3; 4, A).

The L_1 bodies contain phospholipids only, as they give a strong AH+ and PE– reaction (Baker, 1946) and are stained blue by Nile blue (Cain, 1947).

Feyrter's (1936) 'enclosure method' as given by Pearse (1954) stained these bodies metachromatically rose-red. This method, although originally recommended for phosphatides and cerebrosides, has been used to indicate the presence or absence of neutral lipids (triglycerides in this case), as discussed by Pearse (1954). The metachromatic rose-red coloration of these bodies excludes the possibility of the presence of triglycerides in them.

The various extraction tests give a very clear picture of the chemical composition of these lipid bodies (L_1). After cold or hot acetone extraction performed on fresh material, these bodies can be intensely coloured by Sudan black B or acid-haematein. Some of the larger bodies show a clear sign of displacement and aggregation at the two ends of the oocytes (fig. 6, A, opposite p. 326). This is obviously the result of the action of the solvent, which penetrates the oocytes from all sides except the two ends adjoining the inter-follicular regions. This displacement of the lipids is not noticed in the sections of the material previously fixed in formaldehyde calcium. On the other hand when gelatine sections of such material are treated with hot ethanol and ether, the L_1 bodies are mostly dissolved, except that very thin rims appearing in the form of fine crescents, sudanophil and AH-positive, are left over.

When fresh ovarioles are extracted with cold ethanol, the L_1 bodies appear in early stages as very small sudanophil and AH-positive granules, but in larger oocytes the sudanophil granules of fairly large size become aggregated at the ends of the oocytes. A similar picture is given by the treatment with cold and hot ether, except that after cold ether extraction the size of the individual granules is larger than after cold ethanol treatment. These lipid bodies are completely soluble in hot ethanol, methanol / chloroform and methanol / ether.

From these observations it is perhaps reasonable to infer the presence of lecithins (insoluble in ether) and cephalins (insoluble in ethanol), the former being more abundant. But since the solubility tests are not very reliable (Cain

o; Lovern, 1955), such a conclusion may be considered more or less con-
ural.

The second category of sudanophil lipid bodies (L_2) first appear in oocytes
asuring approximately 0·4 mm. They differ from L_1 bodies inasmuch as
y show a colour between pink and blue in the Nile blue test; this suggests
t they have developed some neutral lipids in their contents along with some
dic lipids.

A further elucidation of the chemical nature of the L_2 bodies is provided
the AH test with its PE control. In this test these bodies do not give
homogeneous solid appearance but appear instead as dark blue rings or
scents or even irregular bodies with a completely negative reaction in PE
gs. 4, C; 6, C). This appearance of the L_2 bodies in the AH test is in direct
ntrast with their appearance in various Sudan colouring agents, which
our them homogeneously (fig. 4, B). The co-existing L_1 bodies in these
cytes are homogeneously coloured, even in AH preparations. Thus, it can
ely be concluded that at this stage the L_2 bodies consist of some neutral
ids, completely or incompletely enveloped in a sheath of phospholipids.
he sheath gives these bodies a crescentic or ringed appearance in the AH
t.

Further confirmation of this change in chemical composition is provided
the extraction tests. The neutral lipid contents of the L_2 bodies are dis-
ved by cold or hot acetone extractions either of fresh tissue or of tissue
nply fixed in formaldehyde calcium. Consequently when Sudan colouring
ents are applied to such sections, these bodies give a picture exactly corre-
onding to their appearance in the acid-haematein test. Extractions per-
rmed on fresh tissues, however, do not give such a clear picture of L_2 bodies
in the sections of material fixed in formaldehyde calcium. This is due to
e displacement of these bodies in the oocytes.

The solubility of the neutral lipid contents of the L_2 bodies in cold acetone
ggests that they are either glycerides or cholesterol and/or cholesteryl esters.
he possibility of the latter is excluded as these bodies (L_2) give a completely
gative reaction to Schultz's (1924, 1925) test for cholesterol and its esters
omori, 1952) and to Romieu's modification of Schultz's reaction (Pearse,
54). Some sections were placed in the sun for 10 days in formaldehyde
lcium solution and then immersed for 48 h in 2·5% iron alum; they were
en subjected to Schultz's test. The reaction remained negative. The presence
triglycerides is inferred from the above tests.

The L_2 bodies are completely dissolved even in cold ethanol and cold ether,
spite of their phospholipid contents.

Thus the L_2 bodies have a medullary region rich in rather fully saturated
glycerides, surrounded by a sheath of phospholipids.

Although in oocytes measuring 0·4 mm both L_1 and L_2 bodies are present,
e change in chemical composition of the total lipids of the oocytes is rapid,
d within a very short period of growth the L_1 bodies completely disappear
om the oocytes. In later stages all the lipid bodies, which still show a wide

range in size, are of the L_2 category. Soon, however, the phospholipid sheaths of L_2 bodies attenuate, and simultaneously there is an increase in the number and size of these bodies. Most of these bodies, especially the larger ones, now move towards the periphery of the oocytes.

The L_2 bodies now lose their phospholipid sheaths and thus become converted into L_3 bodies. This change occurs rapidly. The size of most of the lipid bodies increases considerably and they begin to move towards the central region of the oocyte. This change resulting in the attenuation of the phospholipid rims of the L_2 bodies as well as the increase in size is very clearly illustrated in figs. 4, C; 5, A–C; 6, C–E.

It may be noted that L_3 bodies are the only type of lipids present in the oocyte measuring 0·65 mm or more in diameter.

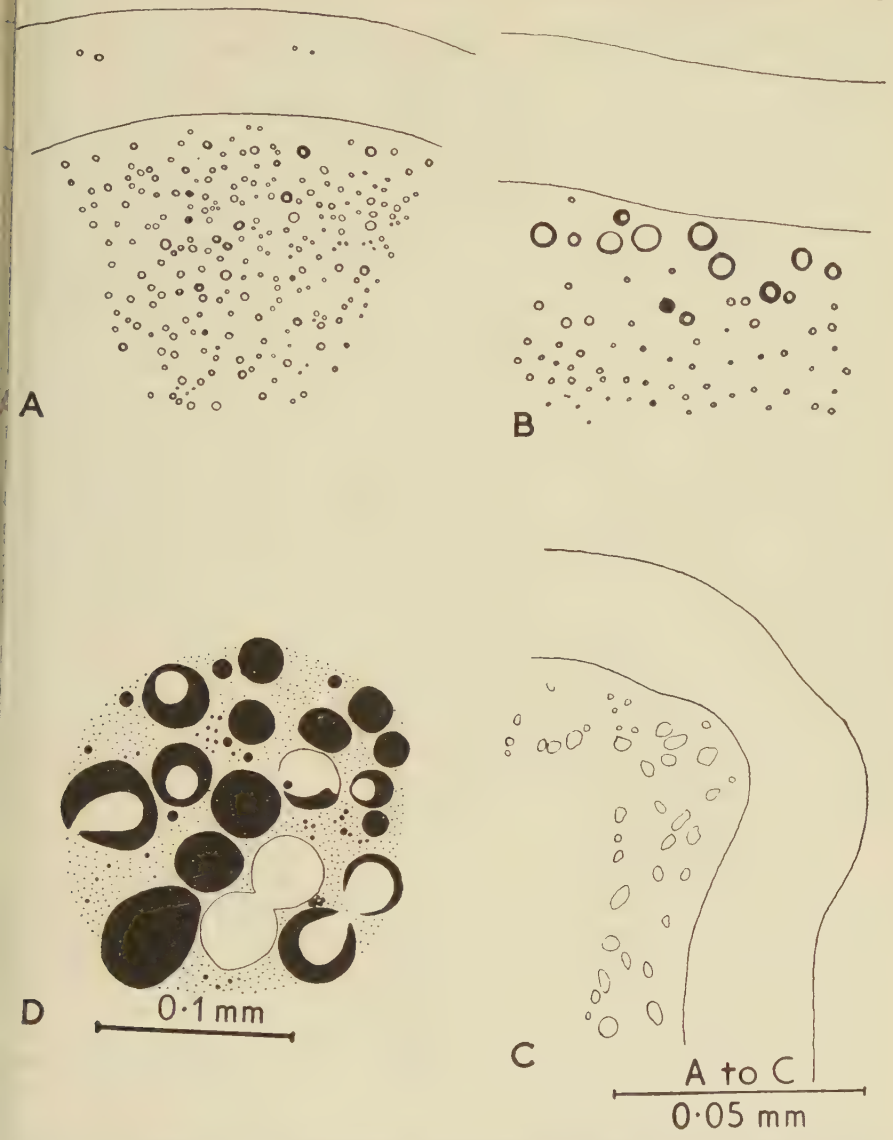
The lipids of the L_3 category generally appear in larger oocytes in the form of spheres ranging in size from very small granules up to about 0·04 mm. Their appearance in Sudan preparations is interesting. When Sudan black B or some other general lipid colorant is applied to the sections of tissue fixed in formaldehyde calcium and postchromed (Baker, 1946), the large L_3 bodies do not colour homogeneously but appear as 'rings' or 'crescents' (fig. 5, D). The smaller L_3 bodies as well as a few of the larger spheres colour homogeneously.

As Bradbury (1956) suggests, this appearance of lipid bodies may be due either to the presence in these bodies of lipids that are solid at room temperature (which varies in Hoshiarpur from 12° C to 40° C during the year), or due to the loss of certain lipids from the spheres during fixation. Bradbury (1956) further showed that in the adipose cells of the leech, *Glossiphonia*, such an appearance of 'fat globules' was due to incomplete fixation in formaldehyde saline, since no 'rings' and 'crescents' appeared in material fixed in formaldehyde calcium.

The appearance of 'rings' and 'crescents' in this material cannot be due to any incomplete fixation, as the ovarioles were always fixed in formaldehyde calcium and postchromed as recommended by Baker (1946, 1956). It is, therefore, reasonable to conclude that this incomplete colouring of larger L_3 bodies was due to the presence of lipids that are solid at room temperature (12° C to 40° C).

That this conclusion is correct has been shown by colouring the gelatine sections of formaldehyde calcium / postchromed tissue in Sudan black B at 60° C for 30 min. Such a procedure eliminated to a very great extent this incomplete colouring of L_3 spheres. Some of them, however, still retained their crescentic appearance, which might be due to the presence of some lipids having a melting-point even higher than 60° C.

As regards the chemical nature of L_3 bodies, they appear bright pink with Nile blue, are completely negative to AH test, and, do not give any metachromatic staining in Feyrter's enclosure method. They are completely soluble in even cold acetone, whether used on fresh or fixed material. All tests for other lipids being negative, the L_3 bodies seem to be composed of



G. 5. A to C, portions of the oocytes from acid-haematein preparations. The drawing shows e gradual attenuation of the phospholipid rims of the lipid bodies. D, a very small portion an oocyte from a Sudan black preparation. The incomplete colouring of the L_3 bodies is shown. (Size of oocytes: A, 0·5 mm; B, 0·63 mm; C, 0·66 mm; D, 1·65 mm).

glycerides, which are not very unsaturated as their reaction to PFAS is eble and they do not become insoluble in fat-solvents after postchroming.

olk globules

When L_3 bodies migrate to the more central areas of the oocytes, some obules of various sizes still appear in the peripheral region. The smallest of

these are minute granules; the largest recorded measures about 0·04 mm diameter. The youngest oocyte in which these globules have been see measured approximately 0·6 mm.

These globules appear for the first time in the form of minute PAS-positi granules in the follicular epithelial cells; thence they migrate to the periphe of the oocyte through the bacterial layer. Suddenly they grow and invade th entire cytoplasm of the oocyte. These granules are negative to all the lip tests tried. Morphologically they are strictly homologous with the 'albuminou yolk' of Nath and Mohan (1929).

The strong PAS-positive reaction of these yolk globules indicates the carbohydrate nature, and this is confirmed by the acetylation and KOH reversal controls (McManus and Cason, 1950). The intense blue colouring i Hg–BPB (Mazia and others, 1953) shows the presence of proteins in them.

A very interesting picture is presented by these globules in material fixe in weak Bouin's fluid and extracted with pyridine in the control to Baker AH test. These globules give a strong blue-black coloration in acid-haematei after this method, but they appear frothy, showing a number of round vacuole in their interior. This frothy appearance is conspicuous even after the PAS an Hg–BPB tests have been applied to these PE sections (fig. 6, F). This froth appearance is also prominent in Carnoy-fixed material stained with PA Since the frothy appearance of these globules is not seen after formaldehyd calcium / postchroming technique, it is possible to attribute it to the loss c some alcohol- or pyridine-soluble material, which, however, is not lipid. W are unable to throw further light on this.

'*Bacteria*' *and lipid synthesis*

Nath and Mohan (1929) and Blochmann (1884, 1887) have describe certain 'bacterioid forms' surrounding the oocytes of *P. americana* and lyin immediately below the follicular epithelium. We have also seen these struc tures, which appear as small rodlets even in the earliest oocytes in th vitellarium.

The various histochemical reactions show that these 'bacterioid forms' con tain phospholipid mixed with free fatty acids. These reactions are positive o

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FIG. 6 (plate). Photomicrographs of gelatine sections of the cockroach ovary subjected t various histochemical reactions.

A, a portion of an oocyte from material extracted with acetone and coloured with Suda black. Note the accumulation of lipid bodies in the corner and the strong positive reaction the 'bacterioid layer'.

B, a portion of an oocyte from material extracted with hot acetone and coloured with Suda black B. The enigmatic sudanophil bodies in the nucleus are shown. L_1 bodies are not i sharp focus.

C–E, portions of oocytes from acid-haematein preparations. The gradual transformation o L_1 and L_2 bodies into L_3 bodies is shown. Note the attenuation of the phospholipid rims Many homogeneous dark bodies in C have become negative to AH in figures D and E.

F, a portion of an oocyte from material extracted with pyridine (control to acid-haematei test). The frothy nature of the yolk globules is shown.

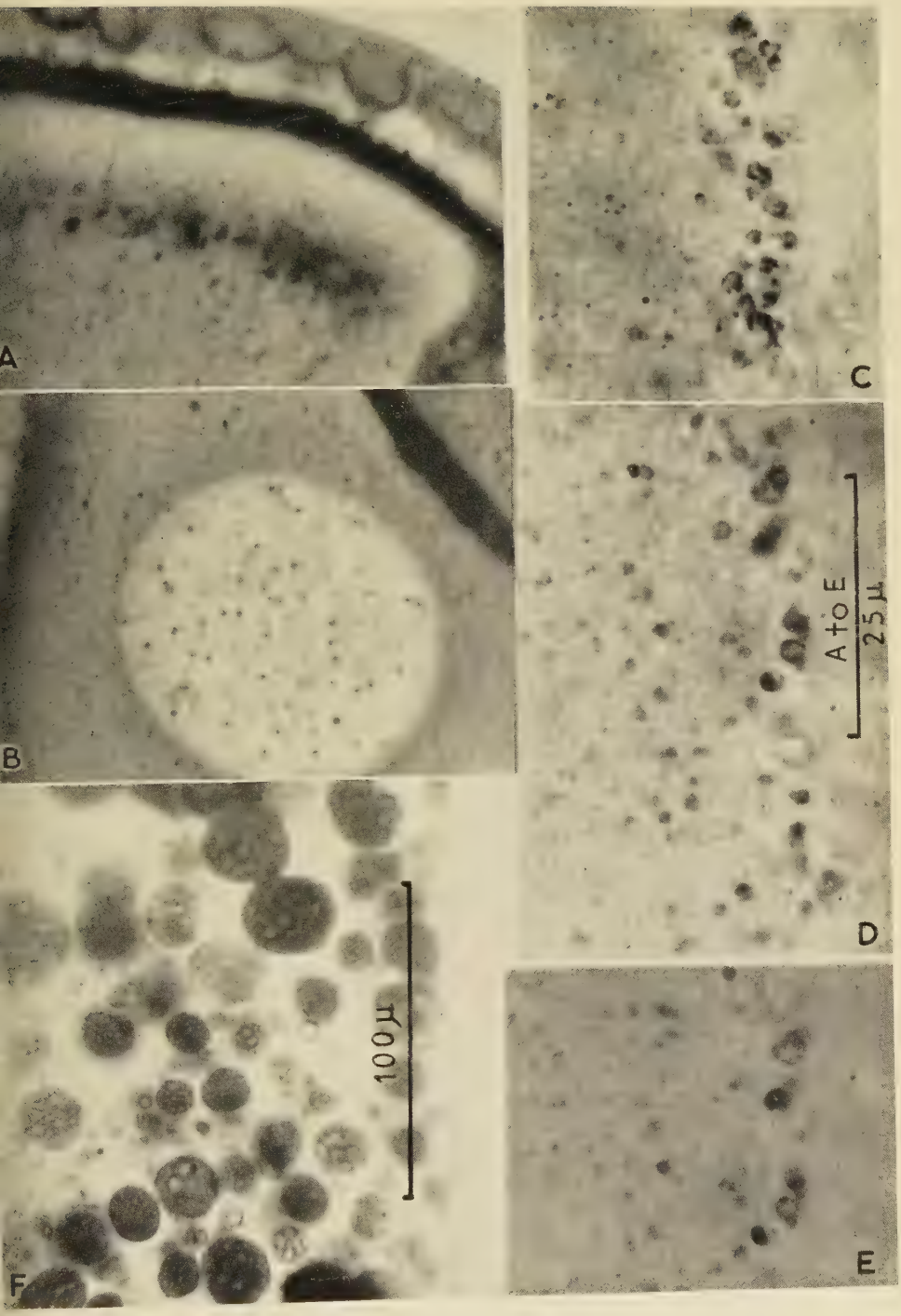

FIG. 6

V. NATH, B. L. GUPTA, *and* B. LAL

e bacterioid forms only up to that stage of vitellogenesis where the L_3 bodies ave not yet fully developed. But once the L_3 bodies reach their maximum evelopment and come to occupy the centre of the oocyte, these 'bacteria' ecome negative to all the lipid tests.

We have not tried any experimental work on lipid synthesis, but the available vidence clearly points towards the conclusion that these bacterioid forms are centre of lipid synthesis in the oocytes. This conclusion is further supported y the fact that the lipid bodies of all categories found in the oocytes of the ockroach generally first appear and mature near this bacterial region. Morever, the presence of free fatty acids also suggests the process of lipid synthesis eing centred in these bodies. It may be repeated that when all the lipid haterial of the oocytes has been synthesized, these bacteria lose their lipid ontents.

It may also be pointed out that lipid synthesis in the ovarioles of *P. americana* nay be divided into three distinct phases:

(1) Phase of phospholipid synthesis, which continues till the oocyte attains size of 0·4 mm (approximately). In this phase the L_1 bodies are formed, and hey increase in number as well as in size.

(2) Phase of triglyceride synthesis, which starts in oocytes measuring pproximately 0·6 mm and continues till they attain a size of approximately ·5 mm. During this period all the lipids synthesized are triglycerides, which o to form the L_3 bodies.

(3) Intermediate phase in oocytes measuring 0·4 mm to 0·6 mm. This hase anticipates the phase of triglyceride synthesis, and during it the phospholipids of the L_1 bodies are either converted into or replaced by triglycerides.

Another important point noticed during the present investigation is that he formation of the L_3 bodies and of other lipid bodies in the oocytes varies considerably according to the season in which the material is fixed. Very arge lipid bodies of the L_2 category have been recorded in oocytes measuring pproximately 0·045 mm in material fixed during the month of April 1957.

Miscellaneous remarks

It has been noticed that the cytoplasm of oocytes measuring more than ·65 mm does not show any definite globules, but is coloured uniformly by various tests, including lipid colorants as well as PAS and Hg–BPB, in sectioned material. This is due to the general diffusion of the material of the various globules under the incomplete and delayed action of the fixatives, which cannot penetrate quickly on account of the development of the chorion. This is confirmed by the fact that when the contents of such oocytes are smeared on a slide and then subjected to the various fixatives and colorants, both types of globules are clearly revealed.

Another enigmatic phenomenon is the appearance of certain well defined sudanophil bodies in the nucleus of comparatively young oocytes after extraction of fresh ovarioles by cold and hot acetone (fig. 6, B), cold ether, cold

TABLE I

Table showing the various histochemical reactions of the oocytes of P. americana

Technique	*Fixative*	*Embedding medium*	*Thickness of sections*	*Reference*	*Diameter of oocytes*	*Nucleus*	*Cytoplasm*	*Mitochondria*	L_1	L_2	L_3	*Bodies in the follicular epithelium*	*Albuminous yolk*	*'Bacterioid forms'*
			(μ)		(mm)									
Sudan black B: in 70% ethanol	FCa and FCa+PC	G	10	Baker, 1949, 1956	0·02–0·40	–	+	+	+ + +	A	A	+ + +	A	++
					0·40–0·60	–	+	+	+ + +	+ + +	A	+ + +	A	+
					0·60–1·65	–	+	+	A	A	+ + + (p)	+ + +	–	–
in 70% ethanol at 60° C	,,	G	10		0·02–1·65	–	+	+	+ + +	+ + +	+ + +	+ + +	–	+ +
in propylene glycol	,,	G	10	Chiffelle and Putt, 1951	,,	–	+ +	+ +	+ + +	+ + +	+ + + (p)	+ + +	–	++
— after treatment with: cold acetone	Fresh or FCa	G	10	Krishna, 1950 Pearse, 1954	,,	+	+	+ +	+ + +	+ + (p)	O	+ + +	–	+
hot acetone	,,	G	10	,,	,,	+	+	+ +	+ + +	+ + (p)	O	+ + +	–	+
cold ethanol	,,	G	10	,,	,,	+	–	–	+	O	O	+ +	–	O
hot ethanol	,,	G	10	,,	,,	–	–	–	O	O	O	O	–	O
cold ether	,,	G	10	,,	,,	+	–	–	+ +	O	O	+ +	–	O
hot ether	,,	G	10	,,	,,	–	–	–	+	O	O	+ +	–	O
methanol / chloroform	,,	G	10	,,	,,	–	–	–	O	O	O	O	–	O
methanol / ether	,,	G	10	,,	,,	–	–	–	O	O	O	O	–	O
Sudan III and IV: in 70% ethanol / acetone	FCa and FCa + PC	G	10	Kay and Whitehead, 1941	,,	–	+	+	+	+ +	+ + + (p)	+ +	–	+
in 1% gelatine suspension	,,	G	10	Govan, 1944	,,	–	+	+ +	+ +	+ +	+ + + (p)	+ +	–	++
Fettrot in 70% ethanol	,,	G	10	Pearse, 1954	,,	–	+	+	+ +	+ +	+ + + (p)	+ +	–	+
Nile blue sulphate	,,	G	10	Cain, 1947, 1948	0·02–0·40	–	B	B	B	A	A	B	A	B
					0·40–0·60	–	B	Nc	B	V	A	B	A	Nc
					0·60–1·65	–	Bk	Nc	A	A	Bl	B		

Acid haematein (AH) after pyridine ext. (PE)	WB + PE	G	10	Baker, 1946	0·02–1·65	+	—	—	O	O	O	O	+ + (p)	O
Plasmal reaction	FCa + PC	G	10	Hayes, 1949	,,	—	—	—	—	—	—	—	—	—
Fischler's reaction	Fs	G	10	Pearse, 1954	,,	—	—	—	—	—	—	—	—	+++
— after treatment with ether / ethanol	FCa or Fs	G	10	,,	,,	—	—	—	—	—	—	—	—	O
Feyrter's enclosure	FCa + PC	G	10	,,	,,	—	—	N	+++(R)	++(R)	—	Nc	—	N
Periodic acid / Schiff (PAS)	FCa and FCa+PC	G	10	Hotchkiss, 1948, Pearse, 1954	,,	—	+	—	—	—	—	+	+++	—
,, ,,	C and Z	P	5	,,	,,	—	—	—	—	—	—	+	+++	O
— after treatment with:														
ethanol	FCa	G	10	Pearse, 1954	,,	—	—	—	O	O	O	+	+++(p)	O
pyridine	WB + PE	G	10	Baker, 1946	,,	—	—	—	O	O	O	O	+++(p)	O
acetylation	FCa + PC	G	10	McManus and Cason, 1950	,,	—	—	—	—	—	—	—	—	—
restoration by 0·1N KOH	,,	G	10	,,	,,	—	—	—	—	—	—	+	+++	—
Mercuric bromophenol blue (Hg–BPB)	,,	G	10	Mazia and others, 1953	0·02–0·60	+	+	++	—	—	A	—	A	—
,, ,,	C and Z	P	5	,, ,,	0·60–1·65	+	+	++	A	A	—	—	+++	—
					,,	+	+	++	—	—	—	—	+++	O
— after treatment with:														
ethanol	FCa and FCa + PC	G	10	,, ,,	,,	—	—	+	O	O	O	O	+++(p)	O
pyridine	WB + PE	G	10	,, ,,	,,	—	—	+	O	O	O	O	+++(p)	O
Performic acid / Schiff (PFAS)	FCa and FCa + PC	G	10	Pearse, 1951 Lillie, 1952	,,	—	+	+	—	—	+	—	—	—
Ciaccio's technique	FCa + PC (1 day)	P	5	Bradbury, 1956	,,	—	—	+	+++	++	N	+++	—	Nc
Cholesterol reactions	FCa and FCa + PC	G	10	Schultz, 1924, 1925 Gomori, 1952 Romieu, 1927 Pearse, 1954	,,	-	—	—	—	—	—	—	—	—

Key: A = absent; B = blue; C = Carnoy; FCa = formaldehyde calcium; Fs = formaldehyde saline; G = gelatine; N = not observed; Nc = not clear; P = paraffin; (p) = partially or ringed; PC = with postchroming; Pk = pink; (R) = rose-red; V = violet; WB+PE = weak Bouin's fluid followed by pyridine extraction; Z = Zenker's fixation for 3 h; + = weak reaction; ++ = moderate reaction; +++ = strong reaction; — = negative; O = dissolved.

ethanol. These sudanophil granules are never seen after fixing in formaldehy calcium and postchroming, nor do they appear after hot ethanol, hot eth methanol / chloroform, or methanol / ether extractions. We are unable to expl this phenomenon, but it might be due to the presence in the nucleus of lip material that is not detectable in ordinary Sudan black or AH preparatio Such a possibility has been pointed out recently by Chayen and others (19 in a number of plant and animal cells.

Discussion

The selective histochemical colouring and solubility tests, carried out gelatine sections of the ovarioles of the cockroach that have been fixed formaldehyde calcium with or without postchroming, reveal the chemic composition and distribution of mitochondria, lipid bodies, yolk globul and the 'bacterioid forms', which are discussed below.

Mitochondria

These are very minute granules, concentrated round the nucleus in t young oocytes, but distributed evenly throughout the cytoplasm in old oocytes. This confirms the observations of Nath and Mohan (1929) in t oocytes of the cockroach.

Nath and Mohan did not work out the chemical composition of the mit chondria, but the present investigations have clearly brought out that t mitochondria of these cells resemble ordinary mitochondria in being co posed largely of protein and phospholipid.

Lipid bodies

The lipid bodies are abundant and widely distributed in the cytoplasm all the oocytes of the ovarioles. By differential histochemical staining ar solubility tests (Krishna, 1950; Pearse, 1954) the following three types lipid bodies have been recorded, which differ from one another in their si and chemical composition.

(1) The first category of lipids or L_1 bodies, which contain phospholip and are homogeneous spheres or granules.

(2) The second category of lipids or L_2 bodies, which are composed triglyceride with a phospholipid sheath.

(3) The third category of lipids or L_3 bodies, which are composed of t glyceride only.

The first type of the lipid bodies correspond to the 'Golgi bodies' of Na and Mohan (1929), but they have not the duplex structure formerly attribute to them. The duplex structure of the 'Golgi bodies' of this cell appears to b due to incomplete reduction of osmium tetroxide.

The second category of lipid bodies, which appear in the oocyte after it h attained a size of 0·4 mm, have undoubtedly a duplex structure and corr spond to the duplex 'Golgi vesicles' of Nath and Mohan (1929).

A hint about their chemical nature is provided by the observations of Nath d Mohan (1929), who have shown that their 'Golgi vesicles' appear solid prolonged osmication, as in Kolatchev preparations, but they again appear plex if such slides are treated with turpentine. This shows that whereas the utral fats of the medullary region are decolorized in turpentine, the lipids the cortical region are not.

The third type of lipid bodies (L_3) correspond to the 'fatty yolk' of Nath d Mohan (1929). They are completely washed out in all the fat solvents, en after postchroming. That the L_3 bodies are composed of triglycerides ly is strongly indicated by the selective staining and solubility tests employed us. These observations are in harmony with those of Nath and Mohan 929), who state that in Kolatchev preparations decolorized in turpentine the tty yolk' spheres appear as clear vacuoles of various sizes, giving the cyto- asm a frothy appearance.

Nath and Mohan (1929) claimed that the 'fatty yolk' in the egg of the ckroach comes directly from the 'Golgi vesicles'. The present investigations e in complete harmony with this claim, as we have already shown that the bodies come directly from the L_2 bodies, and these from the L_1 bodies Golgi bodies').

Finally, it must be emphasized that there is no homology between the lipid dies of the cockroach egg and the classical reticular 'Golgi apparatus', as a ticulum is conspicuous by its absence in this material and the lipid bodies not show any fixed chemical composition.

olk globules

In addition to the lipid bodies there are present in the egg of the cockroach me non-lipid globules or spheres, which appear in later stages of vitello- nesis and seem to be homologous with the 'albuminous yolk' of Nath and ohan (1929) and Gresson (1931). We have shown that these globules contain protein-carbohydrate complex. This is indicated by their positive reactions the PAS and Hg–BPB tests.

These yolk globules seem to arise suddenly as small, PAS-positive granules the follicular epithelial cells and then migrate into the peripheral regions the oocyte. Soon they invade the whole of the cytoplasm, increasing in size nsiderably. There is no evidence of the origin of yolk globules from the ucleolar extrusions' of Nath and Mohan (1929) and Gresson (1931), or om mitochondria.

acterioid forms

We have studied the rodlets called 'bacterioid forms' by Nath and Mohan 929) and Blochmann (1884, 1887). These are situated just below the folli- lar epithelium. They contain free fatty acids and phospholipids. We have t tried any RNA or DNA tests. It seems that the 'bacterioid forms' parti- ate in lipid synthesis in the developing oocyte. This is suggested by the

fact that all the lipid bodies grow in size and number near the layer of br terioid forms, and when the synthesis of lipids is over the bacterioid forms no longer positive to any lipid tests.

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ıe Fine Structure and Morphological Organization of the Peripheral Nerve-fibres and Trunks of the Cockroach (*Periplaneta americana*)

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With three plates (figs. 1 to 3)

Summary

Sections of the peripheral nerve-trunks of the metathoracic leg of the cockroach *eriplaneta americana*) were studied with the electron microscope. Paraffin sections ere also prepared and stained. Protargol succeeds in staining the nerve-fibres. smium tetroxide, a modified Weigert procedure, and Luxol fast blue stain the myelin eaths, as does mercuric bromphenol blue, a protein stain. The axoplasm is relatively ee of formed elements; it contains mitochondria. The myelin sheath, when present the largest and also some smaller fibres, consists of about two or three loose over-oping processes of Schwann cells, covered by their plasma membranes, enclosing oid-like droplets and having a beaded appearance. Between the nerve-fibres in the rve-trunk is Schwann-cell cytoplasm, which arises from Schwann cells that sur-und the whole nerve-trunk. The same fold of Schwann-cell membrane may enter to the formation of the myelin sheath around more than one nerve-fibre. Several nall non-myelinated fibres, which may be as small as 0·3 μ in diameter or less, may e enclosed in the same fold of Schwann-cell membrane. Outside of the Schwann-cell yer and surrounding the nerve-trunk is a thin layer of connective tissue, which does ot send trabeculae into the interior of the nerve. Tracheae and tracheoles accompany e nerve but are not included within the sheaths surrounding a nerve-trunk, even ear the termination of the nerve-fibres in muscle. The structure of the cockroach eripheral nerve is compared with that described by previous investigators, with that other insects, and with invertebrate and vertebrate nerve.

Introduction

MUCH information has recently been accumulated on the physiological mechanisms of nerve and muscle in insects (Hoyle, 1954, *a*, *b*; 1957). rogress in knowledge of the structure of the nerves of insects has not yet aralleled the advances in physiological information. Pringle (1939) has con-ibuted much information on the anatomy and physiology of the leg muscles nd peripheral nerve of the cockroach. In the present investigation, the struc-ıre of the peripheral nerve-trunks and fibres of the cockroach has been ıvestigated by staining techniques for the light-microscope and by electron ıicroscopy. Some information has been obtained about the ultrastructure of ıe nerve-fibres and the relations of the nerve-fibres to each other and their ırrounding sheaths.

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Material and Methods

The metathoracic leg of *Periplaneta americana* was used. Whole legs, pie of leg, and pieces of muscle were fixed in alcohol of varying concentratio osmium tetroxide, or 50% alcohol with 3 g of chloral hydrate per 100 m Cross and longitudinal paraffin sections were prepared. Protargol stains a haematoxylin and eosin were used on the tissues fixed in alcohol. The tissu fixed in alcohol / chloral hydrate were stained by a Cajal block impregnati procedure. Sections were also stained for myelin by a modified Weigert st (Erhart, 1951) and by Luxol fast blue (Klüver and Barrera, 1953), a sulphonat copper salt of phthalocyanine, generously donated by E. I. du Pont Nemours & Co. Attempts were also made to impregnate nerve-fibres formic acid / gold chloride techniques. In addition, the mercuric bromphen blue stain, a procedure specific for proteins (Mazia and others, 1953), w applied to the paraffin sections. Nerve-fibres were also examined under t polarizing microscope.

For electron microscopy, small pieces of metathoracic leg or of musc were fixed for 10–30 min in Dalton's fluid (Dalton and Felix, 1955), a soluti which contains 1% OsO_4, 1% $K_2Cr_2O_7$ at pH 7·2, and 0·85% NaCl. Aft dehydration, specimens were embedded in methyl (1 part) and butyl (6 part methacrylate and ultrathin cross and longitudinal sections (200–300 Å thic were cut with the Servall Porter-Blum microtome. The sections were inserte into an RCA–EMU-type electron microscope. Micrographs were taken a magnification of 2,000 to 6,000 and enlarged to the desired size. The fin magnifications are approximate. Thick plastic sections were cut and viewe under the phase-contrast microscope to provide orientation for the subseque electron microscopy.

Results

Axoplasm

The diameters of the nerve-fibres encountered in cockroach nerve va from about 10 μ to about 0·3 μ or even less. The axons of insect nerve-fibr are usually described as structureless. After the treatment of sections wit osmium tetroxide (fig. 1, B) or after gold or silver impregnation, the axo usually remains unstained. At times a granular precipitate can be seen. Aft staining with mercuric bromphenol blue, the axoplasm is coloured a pale blu (fig. 1, D). Protargol succeeds in impregnating the axons. They are colour

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Fig. 1 (plate). Sections of cockroach nerve. In A–E (photomicrographs) the scale represen 10 μ; in F–H (electron micrographs) it represents 1 μ. Magnifications are approximate.

A, fixed in Dalton's fixative, embedded in plastic. The sheath around the individual nerv fibres is seen.

B, fixed in osmium tetroxide solution. The sheath around individual nerve-fibres blackened.

C, protargol preparation. The axons are impregnated and have a crumpled border.

D, stained with mercuric bromophenol blue. The axoplasm was pale blue. The shea around individual nerve-fibres was also stained.

E, silver impregnation, to show the nuclei in the nerve.

F–G, mitochondria within the nerve-fibres.

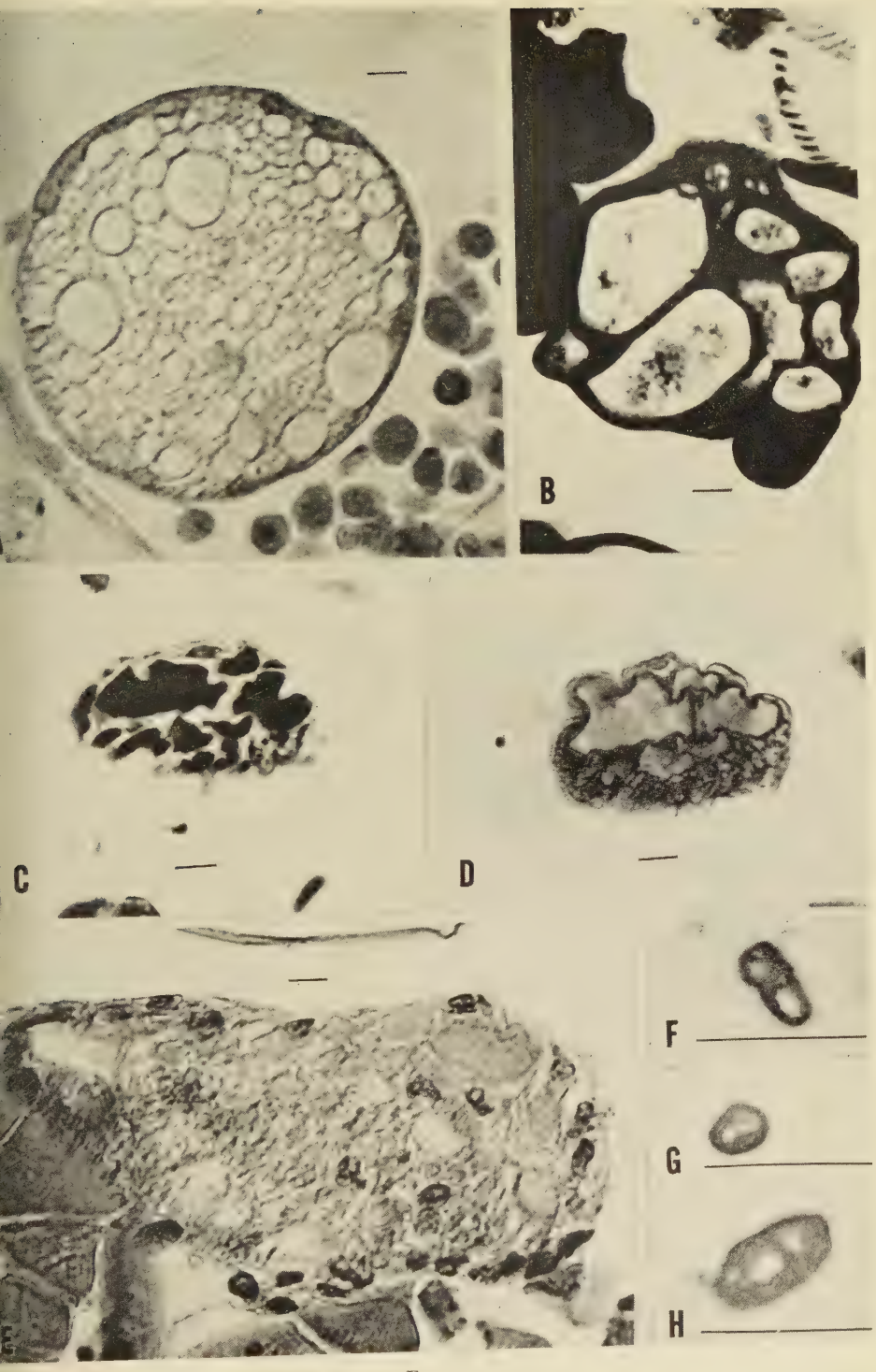

FIG. 1
A. HESS

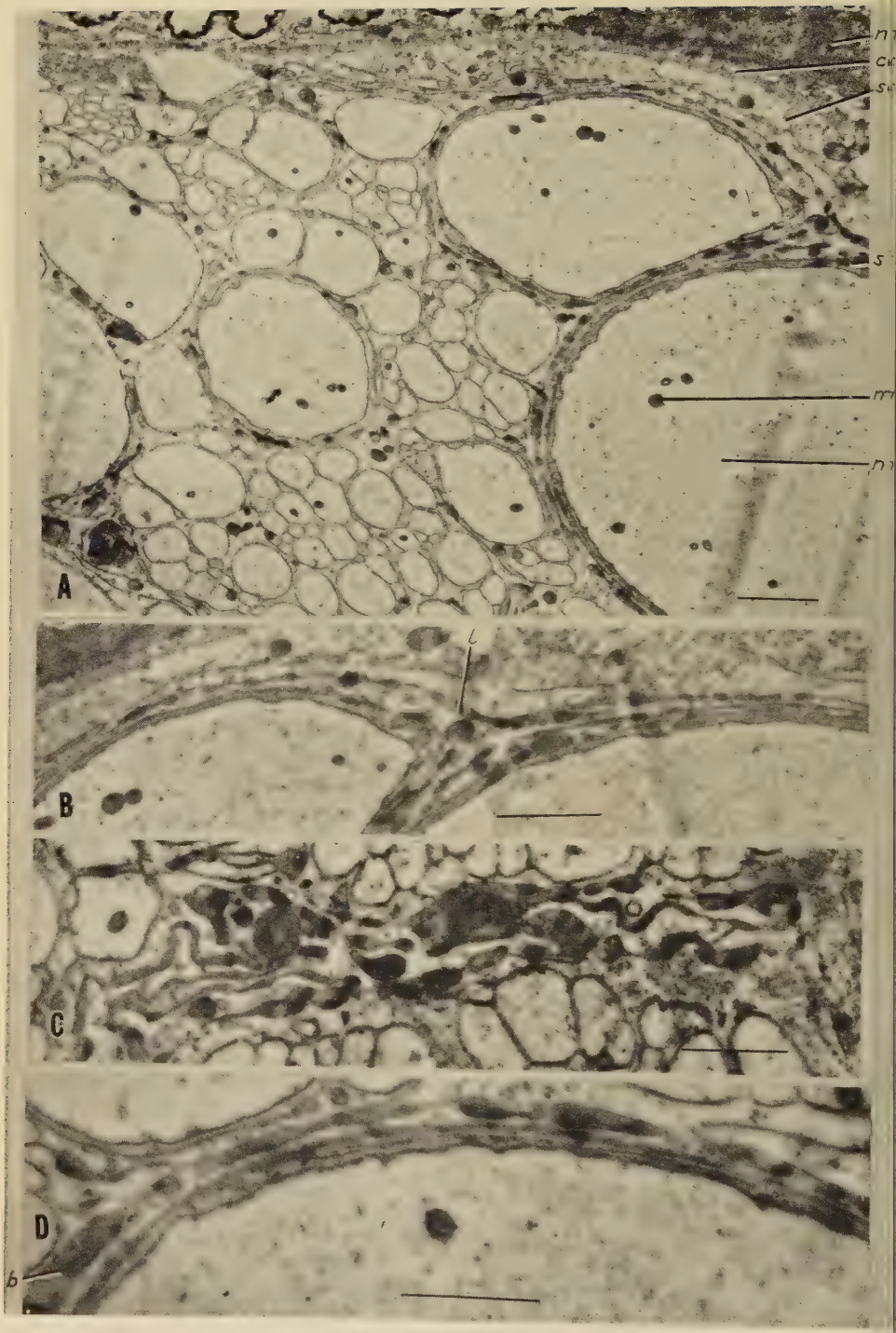

FIG. 2
A. HESS

rk blue or black and frequently appear to lose their round shape and have crumpled border (fig. 1, C).

Electron micrographs reveal the elements present in axons. The most bvious structures seen are electron-dense bodies (figs. 2, A; 3, A) having an ternal structure of folded membranes and frequently having round areas light density (figs. 1, F, G, H). These dark bodies may be mitochondria. In oss-sections of nerve, they usually appear round, which indicates that they e longitudinally oriented in the axon. The rest of the axoplasm usually resents an amorphous granular appearance (figs. 2, A; 3, A). In cross-ctions, some granules have an interior less dense than the periphery, which dicates that they are small tubules longitudinally oriented in the axoplasm. n general, the rather sparse content of organelles and fibrils and light density f the axoplasm are striking, especially when compared with the axons of ertebrates.

heaths of the nerve-fibres

The nerve-fibres rest in the cytoplasm of Schwann cells which enclose the hole nerve-trunk, a relationship that will be described in more detail below. rocesses of these cells, covered by their plasma membranes, twist and turn nrough the interior of the nerve-trunk. Some entwine themselves around a erve-fibre so that it may be surrounded by two or three loosely imbricated rocesses with cytoplasm and its organelles enclosed between the plasma iembrane layers (figs. 2, A, B, D). Electron-dense droplets, apparently of a ipid nature, are frequently seen. These are enclosed between the membranes. These droplets thus enter into the formation of a beaded fatty sheath around ome nerve-fibres (figs. 2, A, B, D), which may be equivalent to the myelin heath of other forms. Most commonly, the largest nerve-fibres are surrounded y this beaded fatty sheath. However, this relationship is not invariable s it is not uncommon to see large nerve-fibres without any membrane folds r lipid or to see small nerve-fibres surrounded by membrane folds and eaded droplets. The membranes appear to course at random through the nterior of the nerve-trunk. At times lipid droplets can be seen enclosed in

FIG. 2 (plate). Electron micrographs of cockroach nerve. The scales represent 1 μ. Magnifications are approximate.

A, cross-section of cockroach nerve-trunk. The axoplasm has an amorphous granular ppearance. The electron-dense bodies in the nerve-fibres are mitochondria. The processes of ne cell surrounding the nerve-trunk (Schwann cell) can be seen entwining themselves around ne nerve-fibres. Some of the membrane wrappings enclose dense droplets, apparently of a pid nature. The wall of a trachea is at the top of the photograph.

B, enlargement of A showing the beaded membrane folds, the enclosed cytoplasm, and roplets forming a sheath on one nerve-fibre and passing on to form a sheath on another erve-fibre.

C, section of a nerve-trunk showing folds of membrane with droplets and not surrounding r forming a sheath on any nerve-fibre.

D, section showing the membrane folds enclosing cytoplasm around a nerve-fibre and the eaded sheath of the nerve-fibre.

b, beaded sheath of the nerve-fibre; *con*, connective tissue layer; *l*, beaded membrane fold assing on from one nerve-fibre to the other; *mit*, mitochondrion; *n*, nucleus of wall of achea; *nf*, nerve-fibre; *s*, sheath of nerve-fibre; *sch*, cytoplasm of Schwann cell.

the membrane and not in relation to any nerve-fibres (fig. 2, C). In additio several nerve-fibres can share the folds of membrane and also the fatty shea Thus, the outer layer of two or three folds of membrane can be seen to lea the original nerve-fibre and join in forming the membrane folds arou another axon (figs. 2, B; 3, E). Nerve-fibres with membrane folds not maki complete turns around them or with only one or two droplets in the membra folds can frequently be seen.

Many fibres are in the cytoplasm without any membrane folds around the It is common to see bundles of very small nerve-fibres enclosed in the sa fold of membrane (fig. 3, D).

That this beaded fatty sheath is equivalent to the myelin sheath can seen by reference to light- and polarization microscope preparations. A myel sheath and the sheath around the cockroach nerve-fibres stain with Sud colouring agents and are birefringent (Richards, 1943, 1944); the latter o servation has also been made in the present study. Both myelin and the coc roach nerve-sheath blacken with osmium tetroxide (fig. 1, B) and also a darkened by Dalton's fluid, as is seen in plastic-embedded sections in th phase-contrast microscope (fig. 1, A). Myelin and the cockroach nerve-shea are also stained by the modified Weigert method employed and by Lux fast blue. The sheath around the cockroach nerve-fibres also stains wi mercuric bromphenol blue, a reagent for protein (fig. 1, D). This probab occurs because of the protein in the sheath contributed by the membran and cytoplasm intercalated between them. Developmental studies are nece sary to determine if the apparently lipid droplets of the membrane folds ari from the cytoplasm and are secondarily included between the membra folds of the Schwann cells or if the droplets are elaborations of the Schwan cell plasma membrane itself.

In a bundle of muscle-fibres one can find the large and small motor-fibr that are destined to innervate the muscle-fibres (Pringle, 1939; Hoyle, 1954 1957) and are near their terminations (fig. 3, C). The complex membran can be seen twisting, apparently at random, between and around the nerv fibres.

Sheaths surrounding the nerve-trunk

Immediately outside the area occupied by nerve-fibres is cytoplasm co taining dense droplets, presumably lipid, large droplets with internal stru ture comparable to mitochondria, and small tubules (figs. 2, A; 3, A). Extenc ing from the plasma membranes covering this cytoplasm are complexly folde membranes which wind their way into the interior of the nerve (figs. 2, A 3, A, B). It thus appears that the sheath-cells send cytoplasmic processes int the interior of the nerve-trunk. These cytoplasmic processes surrounded b membranes form the complex sheath system around the nerve-fibres, whic was described above.

Nuclei can be found in this cytoplasmic sheath or located either at th periphery or in the interior of the nerve (figs. 1, E; 3, A). In the interior of th

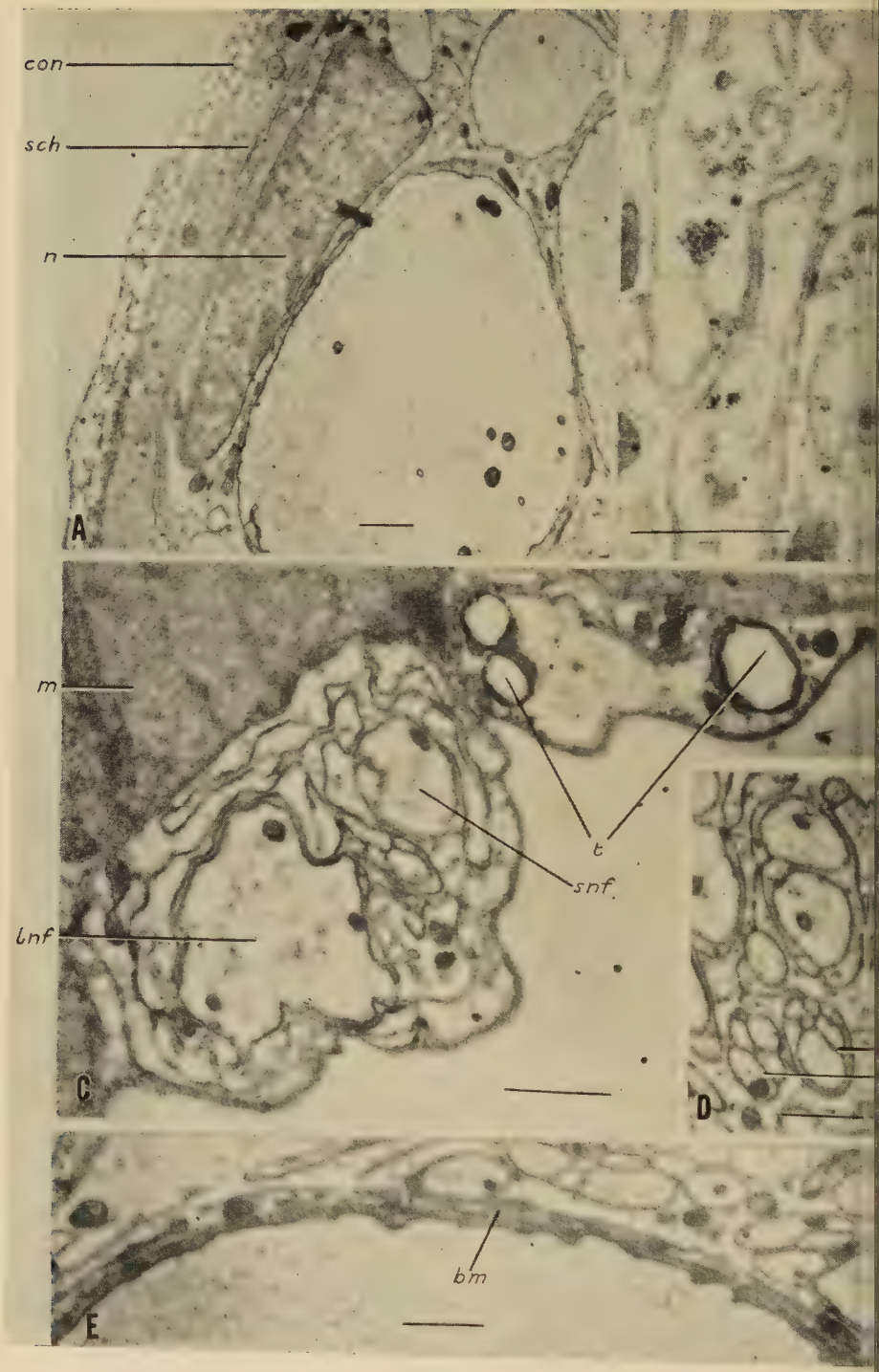

FIG. 3
A. HESS

rve, the cytoplasm is attenuated and interrupted by the foldings of plasma embrane. However, near the nucleus in the cytoplasm or in an oblique sec- n, the cytoplasm is relatively continuous and can be seen best. Many of these lls occur and contribute to the sheaths around the nerve-fibres. The ran- mness of the cytoplasmic strands and membranes in the interior of the rve indicates that the processes of several cells can contribute to the forma- n of a sheath around a nerve-fibre. The arrangement of these cells and their timate relationship to the nerve-fibres indicate that these cells are com- rable to the cells accompanying nerve-fibres in other animals and are hwann cells.

In light-microscope preparations, as, for instance, after protargol staining, e nerve-trunk appears surrounded by a refractile sheath that is unstained part from the nuclei) (fig. 1, C). This sheath stains with connective tissue es and is stained by eosin in haematoxylin and eosin preparations. In electron icrographs, outside the Schwann-cell cytoplasm, short fibrils occur with dications of a periodicity and an appearance reminiscent of connective ssue in other animals (figs. 2, A; 3, A). This layer does not send trabeculae to the interior of the nerve. The nuclei in and on this connective tissue- yer may well belong to cells analogous to fibroblasts. At the interface of the nnective tissue and Schwann-cell layers, a thick membrane occurs and is ade up of the outer limiting membrane of the Schwann cells and the base- ent membrane of the surrounding connective tissue (fig. 3, A).

The above are the sheaths belonging to the nerve-trunk. Outside the thin nnective tissue layer is the haemolymph with its haemocytes. Very fre- uently, nerve-trunks lie beside tracheae and tracheoles. In such instances ne portion of the nerve near the trachea and the wall of the trachea are very intimate relation (fig. 2, A). However, the trachea is never included ithin the connective tissue-sheath of the nerve (fig. 2, A). The same relation olds near the terminations of nerve-fibres. Tracheoles accompany the nerve, ut are not included within the sheaths surrounding the nerve-fibres (fig. 3, C).

FIG. 3 (plate). Electron micrographs of cockroach nerve. The scales represent 1 μ. Magnifica- ns are approximate.

A, cross-section showing the nerve-fibres, showing a Schwann-cell nucleus in the nerve, e Schwann-cell layer, and the connective tissue layer.

B, section through Schwann-cell cytoplasm around the nerve-trunk, showing the complex embrane folds and other organelles.

C, section through muscle, showing the complex foldings of Schwann-cell membrane etween and around the large and small motor-nerve fibres in their sheath. The tracheoles e enclosed in their own cell.

D, section of two bundles of small nerve-fibres. Each bundle is ensheathed by a fold of hwann-cell membrane.

E, section through a nerve-fibre and its sheath of folded membranes and enclosed cytoplasm. he beaded membrane fold of the large nerve-fibre leaves it to encircle partially the small rve-fibre.

bm, beaded membrane fold passing from a large nerve-fibre to a small one; *con*, connective sue layer; *lnf*, large motor-nerve fibre; *m*, muscle; *n*, nucleus of Schwann cell; *sch*, Schwann- ll cytoplasm; *snf*, small motor-nerve fibre; *t*, tracheoles; *x*, *x*, two bundles of small nerve- res, each bundle ensheathed by a membrane fold.

Discussion

The description of the structure of cockroach nerve in electron micrograp conforms very well to that of previous investigators who employed light- a polarization microscopy. The structure of the sheath around the individu nerve-fibres described in the present investigation may well be responsible f the optical properties of this sheath, as described by Richards (1944). Th the protein of the individual nerve-sheaths of insects may be collageno (Richards, 1944) seems to be incorrect.

The sheaths around the nerve-trunk also require comment. The nerv trunk is usually described as ensheathed by an outer homogeneous lay called the 'neural lamella' and an inner cellular sheath denoted as the 'per lemma' (Hoyle, 1952). The 'perilemma' is equivalent to the Schwann-c layer and the 'neural lamella' appears to be the connective tissue-sheat Richards (1944) suggests that the neural lamella does not seem to be collag 'since it does not swell, dissolve or even lose its birefringence in dilute ace acid (3 days) and since immersion experiments give different results for t neural lamella and the presumably collagenous sheaths around individu nerves'. However, as was mentioned above, the sheaths around individu nerve-fibres are probably not collagenous; the neural lamella appears to be s The descriptions of the nerve-sheaths of insects by Scharrer (1939) a Twarog and Roeder (1956) are restricted to the ganglia. Contrary to gener belief, the composition of the sheaths on the ganglia and those around t nerves differ (Hess, 1958); hence discussion of the sheaths around the gangl will be reserved.

There appear to be many differences in structure between the peripher nerve of locusts, as described by Hoyle (1954*a*), and that of cockroaches, described here. The locust nerve apparently has some kind of connecti tissue in its interior, the cockroach has Schwann-cell cytoplasm between i nerve-fibres. The locust nerve-trunk is surrounded by a tracheolated men brane and a fatty envelope, that of the cockroach is not. Hoyle (1957) includ a trachea within the outer sheath of locust nerve near a motor end-plat Although I have not yet studied neuromuscular endings, the tracheoles ne nerve terminations are not included within the outer sheaths of the nerv Whether these differences in structure between locust and cockroach ner are real or whether they will break down upon more detailed study remai to be seen.

In lobster and squid nerve-fibres, osmiophil dense-edged layers, similar the membrane foldings of the Schwann cell described here in insect nerv occur at the axon interface and in the cytoplasm of the Schwann cell (Gere and Schmitt, 1955). However, the insect nerve usually has in addition lipi like droplets enclosed or intercalated between the membrane folds of Schwa cell that surround the nerve-fibres; thus the insect myelin sheath is common beaded in appearance.

The differences in the relation between cockroach nerve-fibres and Schwa

lls and between vertebrate axons and Schwann cells are interesting. In rtebrate nerves, each myelinated nerve-fibre is enclosed by a Schwann cell, hile several non-myelinated fibres share a Schwann cell (Gasser, 1955; Hess, 56). In the cockroach, Schwann cells surround the whole nerve-trunk. ach vertebrate non-myelinated fibre is suspended in the cytoplasm of the me Schwann cell by a mesentery of Schwann-cell membrane called a nesaxon' (Gasser, 1955; Hess, 1956). The 'mesaxon' of insect nerve-fibres n surround several small non-myelinated fibres, which are included as a undle in the same Schwann-cell membrane. The myelin sheath of mammalian erve-fibres consists of concentric lamellae wrapped around the axon (Hess nd Lansing, 1953). It has been suggested that these lamellae are formed by rappings of the Schwann-cell membrane (Geren, 1954). The insect fatty heath is also apparently formed by wrappings of the Schwann-cell membrane, hich form lamellae on the nerve-fibre. The insect lamellae are not so tightly acked and cytoplasm intervenes between them. In this respect, the insect tty sheath bears a striking resemblance to a developing immature vertebrate yelin sheath. The fatty sheath of insects includes droplets apparently of lipid nature in addition to the wrappings of Schwann-cell membrane and is eaded in appearance. The lamellae of the vertebrate myelin sheath are of even nickness and consist only of wrappings of the Schwann-cell membrane. The rappings of the Schwann-cell membrane in vertebrates are restricted to ndividual nerve-fibres so that most commonly two myelinated axons do not nare a myelin sheath and are not included within the same Schwann cell, lthough rarely the latter has been seen to occur (Hess, 1956). In insects, everal myelinated and non-myelinated fibres share the same Schwann cells nd the wrappings of the same Schwann-cell membrane can frequently be een to leave one nerve-fibre and join in the formation of the beaded fatty heath of other fibres, so that several nerve-fibres can be seen to share in the rappings of the same Schwann-cell membrane and fatty sheath. The appar-nt randomness of the foldings of the Schwann-cell membrane in insect nerve s striking. Perhaps a study of the development of insect peripheral nerve-runks will reveal that the sheath system of their constituent nerve-fibres is ot as random as it appears in mature forms.

The sheaths of the cockroach nerve-trunk and those of vertebrate nerve are omparable. The connective tissue layer surrounding the whole nerve-trunk f the cockroach nerve is analogous to that of the epineurium of vertebrate erves. However, this layer does not send trabeculae into the interior of the erve and hence, at least for cockroach nerve, there is no peri- or endo-eurium. The thick membrane at the interface of Schwann-cell and connective ssue layers and composed of the outer limiting membrane of the Schwann ells and the basement membrane of the connective tissue is reminiscent of he neurilemma or Schwann sheath of vertebrate nerve, which is made up of imilar membranous layers (Hess, 1956). There are also close functional arallels in the sheaths investing insect and vertebrate nerve tissue (Hoyle, 953; Twarog and Roeder, 1956).

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Observations on the Non-calcareous Component of the Shell of the Lamellibranchia

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Summary

comparison has been made between the staining reactions and histochemical perties of the non-calcareous material (conchiolin) from the different layers of ls of *Anodonta cygnea*, *Mytilus edulis*, and *Ostrea edulis*. Acid hydrolysates of the chiolin protein have been analysed qualitatively by paper chromatography.

he composition of the conchiolin in *Anodonta* confirms the view that correspond- layers of the valves and ligament represent modifications of the same layers of the l. In this bivalve, the properties of the outer layers of the valves and ligament are ely comparable with each other, and also with those of the periostracum and the on layer of the ligament. All these regions consist of a quinone-tanned protein, rolysates of which are rich in phenolic amino-acids, especially tyrosine, and in cine.

Much of the periostracal conchiolin in *Mytilus* shows basically the same properties he periostracum in *Anodonta*. However, the outer layers of the valves and ligament Ostrea and *Mytilus* each exhibit progressively greater specialization compared with situation in *Anodonta*. This is most marked in *Mytilus* where these components er completely in character.

The conchiolin in the inner shell layers differs markedly in composition from that the outer layers in *Anodonta* and *Ostrea* and from the periostracum in *Mytilus*. drolysates of its protein constituent contain appreciably more aspartic acid and tamic acid but much smaller amounts of phenolic amino-acids. The protein is only ıtly tanned. Although in these properties the corresponding inner layers of the ves and ligament appear fundamentally alike, each component has certain specialized tures. It is suggested that the modifications shown by the protein of the inner ment layer, which is characterized by a high content of proline and methionine, correlated with the specialized function of this region of the shell.

Introduction

T has been maintained on morphological grounds that, in the Lamelli- branchia, the outer and inner layers of the valves and of the ligament are be regarded as representing local modifications of the same two layers of shell (Owen, Trueman, and Yonge, 1953). However, little attempt has been made to determine to what extent the non-calcareous components these corresponding layers of the valves and ligament are comparable mically. This non-calcareous material, originally termed conchiolin by my (1855), makes up the bulk of the ligament and is also a variable but portant constituent of the valves. Trueman (1949) has shown that in *lina tenuis* the conchiolin in the outer layer of the ligament differs in com- ition from that in the inner layer, and that these two types of conchiolin pear to correspond respectively with those of the periostracum and the er complex layers of the valves. He considered, however, that the chemical

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properties of the various forms of conchiolin could be more effectively studi in bivalves which have larger ligaments and less highly calcified valves than the case in *Tellina*.

In this paper, the properties of the non-calcareous material in three su bivalves are described. Particular reference is made to the shell conchiolin *Anodonta cygnea*, the properties of which have already been outlined (Bee ham, 1954), and additional observations are made on *Mytilus edulis* and *Ostr edulis*. The staining reactions and histochemical properties of the non-calcareo components of the different layers of the shell are compared in detail a their protein contents analysed qualitatively by means of paper chromat graphy. Sections of the conchiolin were prepared from shells fixed in Bouir fluid, 4% aqueous neutral formalin, or other routine fixatives, and decalcifi in dilute hydrochloric acid. Ester wax (Steedman, 1947) was found to be t most suitable embedding medium, although in the case of the ligament, whi tends to become extremely brittle during wax embedding, sections were oft cut directly on a freezing microtome.

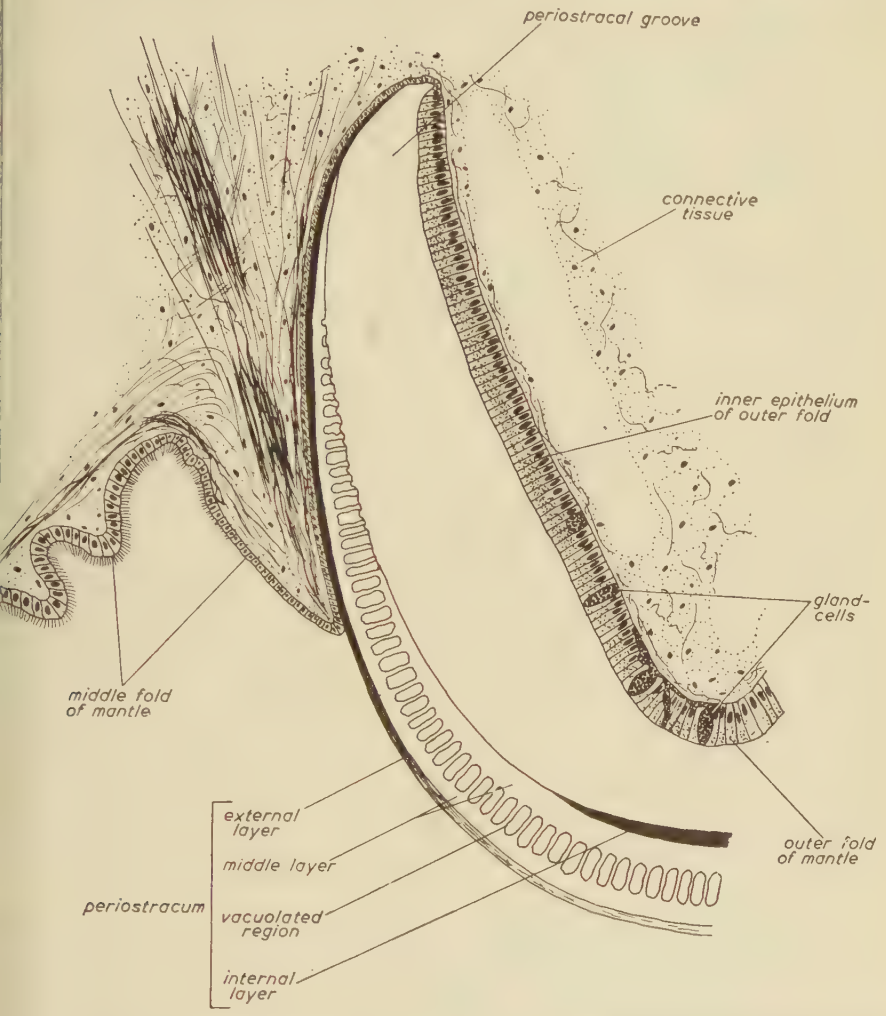
Staining Reactions

The non-calcareous components of the outer and inner calcareous laye of the valves in *Anodonta* and *Ostrea* can readily be differentiated in secti both by their appearance, owing principally to the well-marked prismat structure of the former, and by their reactions to triple stains. With Mallory or Masson's stains, the outer layer always colours red and the inner layer bl or green respectively. The thin, whitish laminae of the inner layer are al distinguished by the fact that they colour relatively more strongly wi Delafield's or Ehrlich's haematoxylin and show slight metachromasia wi aqueous toluidine blue, although these differences between the layers are le pronounced in *Ostrea* than in *Anodonta*. The outer layer in *Anodonta* is co tinuous with the overlying periostracum, up to 15 μ thick, which has a natur amber colour and is refractory to stains. In contrast, the extremely th periostracum in *Ostrea* is hardly distinguishable overlying the shell, and i properties are not recorded here.

Although the outer and inner calcareous layers of the valves in *Mytil edulis* differ in crystalline structure (Field, 1922; White, 1937), their co chiolin components have a similar appearance in section. Unlike the corr sponding regions in *Anodonta* and *Ostrea*, they have the same stainir reactions and both colour blue with Mallory. The whole of this ground sul stance of the valves is sharply distinguished in structure and properties fro the superficial thick periostracum.

The periostracum in *Mytilus*, which is secreted by the inner epithelium the outer fold at the mantle edge, consists basically of three layers (fig. 1). T avoid confusion with the main layers of the shell, these will be referred to the external, middle, and internal layers. The thin external layer is formed the extreme base of the periostracal groove. At its origin this layer sho affinity for the acid fuchsin in Mallory's stain, but the reaction fades as t

ternal surface of the periostracum comes into contact with sea-water (fig. 1). ne middle layer is secreted next; it constitutes the bulk of the periostracum d is up to 80 μ thick. It consists of clear, yellowish conchiolin, mostly un-loured by routine stains, in which lies a central vacuolated region (fig. 1).



IG. 1. Diagram of a transverse section through the mantle edge of *Mytilus edulis* to show the structure of the periostracum. Not to scale.

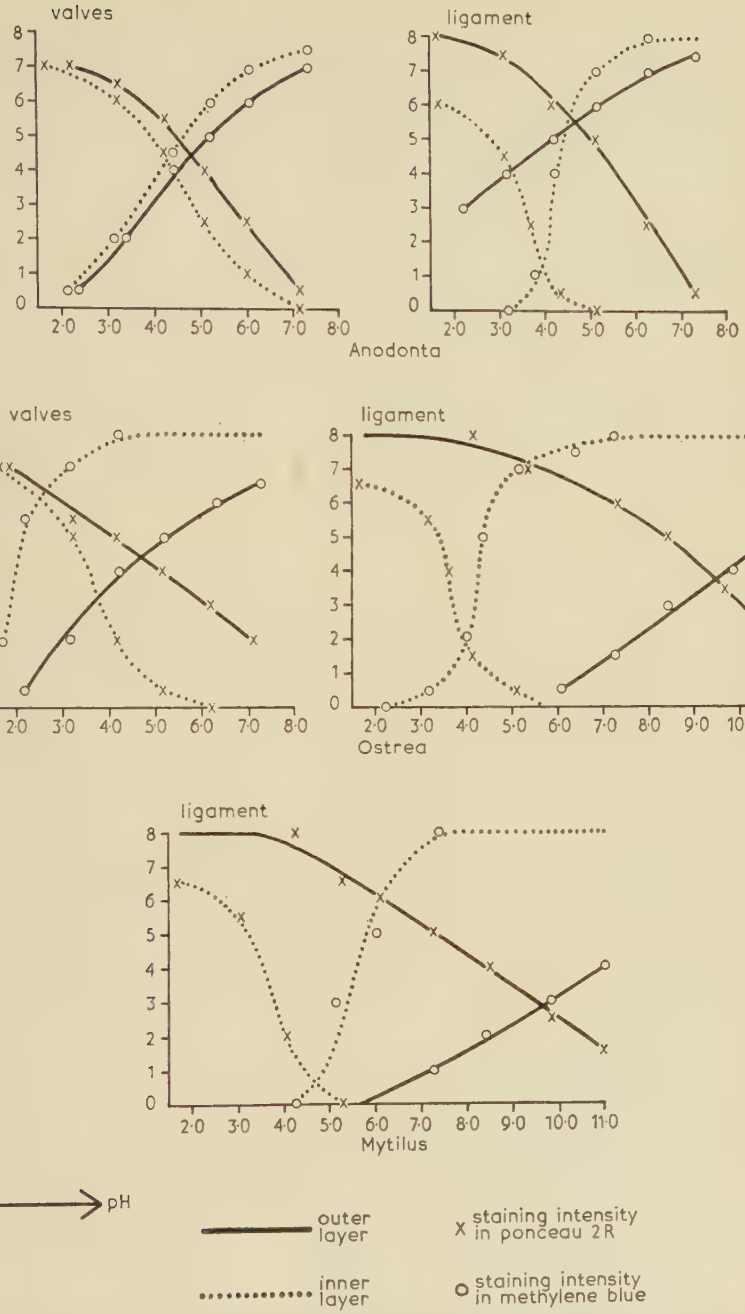
inally, the internal layer is deposited by the epithelium towards the tip of ne outer fold. It has a natural brownish colour and in both Mallory and Iasson preparations it stains deep red. The products of the underlying gland-ells in this region of the outer mantle fold (fig. 1) are considered to have a ubricatory function (Beedham, 1958), and do not appear to be directly con-erned in periostracum formation.

As in other lamellibranchs described by Trueman (1950*b*), the outer an inner layers of the ligaments in the bivalves investigated are rapidly diffe entiated by Mallory, the former colouring with the acid fuchsin and the latt with aniline blue. A similar distinction is obtained with Masson's trichrom stain. The inner layer is also comparable with the corresponding region the valves in that it shows affinity for Ehrlich's haematoxylin and exhibi metachromasia with toluidine blue. In contrast, the periostracum which ex tends over the ligament in *Mytilus* (Yonge, 1955) and the amber-coloure fusion layer of the ligament in *Anodonta* (Beedham, 1958) are refractory t most staining techniques.

The characteristic reaction of the conchiolin to triple stains suggests th the outer and inner layers differ in composition and that, in certain lamell branchs, corresponding layers of the valves and ligament have basically th same properties. Trueman (1951) observed, moreover, that these reaction emphasize the homologies of corresponding layers of the shell in differe bivalves. Consequently, it seemed worth while to investigate the stainin properties of the shell layers further by comparing their reactions to specifi acid and basic dyes in a series of buffer solutions (fig. 2). Although Levin (1940) has shown conclusively that this technique of staining at controlled pH does not, as had been previously claimed, determine the isoelectric points components of tissue sections, it remains, nevertheless, a useful method for com paring the staining properties of different elements within the same section.

Since differentiation of the shell layers with trichrome stains is invariabl obtained whatever the method of fixation, the observations were made unde the same conditions on material preserved in routine fixatives. Sections wer stained for 24 to 36 h in 0·0001 M solutions of the acid dye (ponceau 2R) an the basic dye (methylene blue) in standard buffers ranging from pH 1·6 t pH 11·0. The intensity of staining of the conchiolin was estimated arbitrarily on the scale 0 to 8 by visual comparison with colour charts prepared wit solutions of ponceau 2R and methylene blue of different concentration (Levine, 1940). The values obtained were plotted on graph paper with stain ing intensity on the ordinate and pH of the dye-buffer solutions on th abscissa (fig. 2).

The results show that, in general, the outer layers of both valves an ligament always stain more strongly with the acid dye than do the inne layers (fig. 2), a feature which can be correlated with the greater affinit which they show for the acid fuchsin in Mallory's stain. However, this differ ence varies considerably in extent. It is most pronounced in the ligaments *Mytilus* and *Ostrea*, in which the outer layers stain intensely with poncea 2R over a very wide pH range (fig. 2). In the valves of *Anodonta*, on the othe hand, the difference in acidophily is small, although it is interesting to not that the distinction between the layers can be enhanced by treatment wit tap-water. This procedure, which is normally adopted during Mallory's tech nique, causes rapid decolorization of the inner layer, whereas the acid dy tends to be retained by the outer layer.



G. 2. Diagram showing the staining reactions at controlled pH of the outer and inner layers the shell in *Anodonta*, *Ostrea*, and *Mytilus*. Intensity of staining in the acid dye, ponceau 2R, marked with a cross and that in the basic dye, methylene blue, with a circle. The buffers ed, namely, Walpole's sodium acetate / HCl (below pH 2·2), Clark and Lubs's standard ffers (pH 2·2 to pH 10·0), and Sörenson-Walbum's glycine and NaCl/NaOH (above I 10·0), were all employed at one-tenth their standard concentration. Staining curves drawn th unbroken lines represent the outer layers of the valves and ligament; those with broken lines the inner layers.

A similar variation is observed in the degree of differentiation obtain between the shell layers with methylene blue (fig. 2). The difference in ba philia between the layers in *Anodonta* is not unusually large, but in the valv of *Ostrea*, the inner layer is sharply distinguished from the outer, and al from the rest of the conchiolin investigated, in that it shows marked affini for the basic dye. In *Mytilus* and *Ostrea*, the outer and inner layers of t ligament are similarly well defined, but this is due to the exceptionally we reaction of the former to methylene blue (fig. 2).

The difference in staining properties which undoubtedly exists betwe the outer and inner layer conchiolin is, therefore, subject to some variatio As a result, the reactions of corresponding layers of the valves and ligame may appear somewhat similar, as is the case in *Anodonta*, or they may sho appreciable differences, as in *Ostrea* (fig. 2). It is evident that the properti of individual layers can differ from those of their homologues both in the sa shell and in the shells of other bivalves, these modifications not necessari being revealed by Mallory's and other routine staining techniques.

Histochemical Properties

Anodonta cygnea

The chemical and histochemical reactions of the different layers of t shell are compared in detail in table 1. As previously observed (Beedha 1954), the conchiolin consists mainly of protein, although the colouring of t outer and inner layers with Sudan black and their weak reaction to the period acid / Schiff test (Hotchkiss, 1948) indicates respectively that lipid materi and possibly some polysaccharide may also occur. The mucin stain, alci blue (Steedman, 1950), reacts with the inner layers but not with the remaind of the shell conchiolin. It is interesting to observe that fragments of conchiol from certain parts of the shell resist treatment with hot, saturated potassiu hydroxide and react to the chitosan / iodine test for chitin (Campbell, 192 (table 1). Chitin has already been recorded in the shell of *Anodonta* and oth bivalves by Wester (1910). Nevertheless, even if chitin occurs it is prese only in very small quantities. Analyses by Schlossberger (1856), Wetzel (190 and many others have shown that conchiolin has a nitrogen content approximately 16% in contrast to 6·5% for chitin, which indicates that i principal constituent is protein.

When treated with a series of reagents devised by Brown (1950*b*) for dete mining the types of linkages present in structural proteins, the whole of t non-calcareous material in the shell dissolves almost completely in the sodiu hypochlorite reagent only (table 1). This suggests that much of the conchiol protein is hardened by quinone-tanning. As is well known, the presence quinone-tanned proteins has been established in many invertebrate skelet structures, including the shell and byssus of certain lamellibranchs (Brow 1950*a*, 1952; Trueman, 1950*a*), and also in the egg capsules of selachia (Brown, 1955; Threadgold, 1957).

The outer layers of the valves and ligament, which have remarkably simil

TABLE 1

Summary of the results of chemical and histochemical tests on the non-calcareous material in the different layers of the shell of Anodonta cygnea

Intensity of the reaction is represented arbitrarily by number of Xs; *tr* indicates trace amount; O indicates no recognizable response. Details of the tests used are given in the text

Test	*Valves*			*Ligament*		
	Perio-stracum	*Outer layer*	*Inner layer*	*Fusion layer*	*Outer layer*	*Inner layer*
Cl, conc., room temp., 8 h	All persist					
Cl, conc., 55° C, 8 h	Persists		Slowly dissolves	Persists		Quickly dissolves
OH, hot, sat. .	Dissolves		Fragments persist	Fragments persist		Dissolves
% sodium hypochlorite	All mostly dissolve					
dium sulphide	No apparent effect					
llon . .	XXXXX	XXXX	X	XXXXX	XXXX	X
nthoproteic .	XXXXX	XXX	X	XXXXX	XXXX	X
lin (Baker) .	XXXXX	XXXX	tr	XXXXX	XXXX	X
gentaffin .	XXXXX	XX	? tr	XXXXX	XXX	X
kaguchi (Baker)	XX	XXX	XXX	X	X	XXX
lphur . .	tr		O	tr		XXXX
dan black B .	tr	XXX	XXX	tr	XX	XX
riodic acid / Schiff	O	? tr	X	O	X	X
cian blue .	O	O	XXX	O	O	XX
itosan (Campbell)	O	O	+ve	+ve		O

stochemical properties, both differ markedly from the inner shell layers in eir reactions to tests for phenolic groups (table 1). The outer layers and, to even greater extent, the periostracum and fusion layer always react strongly the Millon and xanthoproteic tests and to Baker's (1956) modification of lin's method for phenols, which indicates that all these regions of the shell ntain a large proportion of phenolic groupings and are highly tanned. As ll be shown later, hydrolysates of the protein contents of these regions con-n considerable amounts of phenolic amino-acids, especially tyrosine.

As observed by Trueman (1950*a*), the presence of an orthoquinone which ay be responsible for hardening the conchiolin is suggested by the fact that, en after boiling, sections of the outer layer of the ligament are still able oxidize the Nadi reagent (dimethyl-*p*-phenylenediamine and α-naphthol). owever, attempts to detect, either in the conchiolin or in the mantle tissues, pecific polyphenol which might be the precursor of the tanning agent were her unsuccessful. The argentaffin test (Lison, 1953), which has been widely ed to indicate the localization of polyphenols in quinone-tanning systems, cts moderately with the outer layers and intensely with the periostracum d fusion layer (table 1), but this reaction, which is also given by the

epithelial cells of the outer mantle fold (Beedham, 1958), is not specific. More over, the response of the conchiolin to the argentaffin and other phenoli tests is apparently unaffected by the prolonged treatment with acid, alcohol &c., involved in the preparation of decalcified sections, which suggests tha it is due to firmly bound aromatic groups rather than to free polyphenol The chromaffin test for polyphenols (Lison, 1953) reacts with the fusion an outer layers of fresh undecalcified sections of the ligament, but the mor specific potassium iodate reaction, and the ferric chloride and ammoniur molybdate tests for orthodiphenols (Lison, 1953), were found to give mainl negative results.

A free orthodiphenol may, of course, occur in *Anodonta* but not in sufficien quantities to be detected histochemically. Possibly this orthodiphenol initially present in the tissues in a masked condition, as is known to occur i the case of protocatechuic acid, the precursor of the tanning agent in th oothecae of *Blatta* and *Periplaneta* (Brunet and Kent, 1955), although th would not appear to explain the failure to locate free polyphenols in th immediate vicinity of the shell. However, the presence of an enzyme capab of oxidizing polyphenols is indicated by a number of techniques. If fres sections of the ligament are incubated with *l*-tyrosine in buffer at pH 8·0, th incubation medium slowly darkens and laminae of the fusion layer and par of the outer layer turn brownish black. This reaction, which is inhibited b potassium cyanide or by boiling, indicates the occurrence of a thermolabi polyphenol oxidase (Brown, 1952). Smyth's (1954) catechol technique for th detection of polyphenol oxidase also reacts positively with these and certai other regions of the ligament.

Although it can be assumed from these results that the conchiolin in th periostracum and in the fusion and outer layers consists largely of a quinon tanned protein, the nature of the tanning process is obscure. The rich tyrosi content of the protein indicates, as already suggested by Roche, Ranson, an Eysseric-Lafon (1951) in observations on the valve conchiolin of certai lamellibranchs, that hardening may be partly effected by the tanning actio of an orthoquinone produced by the oxidation of the side chains of th tyrosine component. It has been suggested that this form of aromatic bondin in which a phenolic protein acts both as substrate and as tanning agent, i. undergoes 'self-tanning', occurs in the byssus of *Mytilus* (Brown, 1950*a*, 195 Smyth, 1954) and the cuticles of myriapods and insects (Blower, 195 Dennell and Malek, 1956). However, theargentaffin reaction of the conchiol indicates the presence, especially in the periostracum and fusion layer, of more powerful reducing agent than a phenolic protein. Dennell and Mal (1955*b*) suggest that such a reaction could well be caused by oxidation pr ducts of polyphenols. They demonstrated that fully hardened cuticles *Periplaneta americana* still give an intense argentaffin reaction even after free dihydroxyphenols present have been extracted. It is provisionally su gested, therefore, that the phenolic protein in the periostracum, fusion laye and outer layers is initially 'self-tanning', but that at least part of it su

ıently undergoes phenolic tanning in the manner visualized by Pryor
ıo, *a*, *b*). The situation in *Anodonta* may be analogous to that in the cuticle
Periplaneta, in which tanning is considered to occur in two such stages
nnell and Malek, 1955, *a*, *b*, 1956). Whatever the tanning method, there
ttle doubt that final hardening is most pronounced in the amber-coloured
chiolin of the periostracum and fusion layer, rendering it refractory to
ns.

n contrast to the conchiolin described above, that in the inner layers of the
es and ligament reacts weakly to all tests for aromatic groupings (table 1).
se properties, coupled with the fact that hydrolysates of the inner layers
tain only small amounts of phenolic amino-acids (see below), suggests
the inner layer protein is relatively lightly tanned. The difference in
ility between this protein and the highly tanned component of the remain-
shell layers is illustrated by the reaction of the conchiolin to concentrated
neral acids. Although all the shell conchiolin shows some resistance to
centrated hydrochloric acid at room temperature, that in the inner layers
olves more quickly when the acid is heated (table 1).

n these and other properties, the conchiolin in the inner layer of the valves
responds closely with that in the inner ligament layer. Both these regions
ct moderately to Baker's (1947) modification of the Sakaguchi test for
inine, and are distinguishable in this respect from the fusion and outer
ers of the ligament (table 1). However, the inner layer of the valves differs
m its homologue in that it responds to the chitosan reaction, whilst the
er ligament layer is characterized by the fact that it reacts positively to the
t for sulphur described by Hawk, Oser, and Summerson (1954) (table 1).
tions of conchiolin from each shell layer were heated with potassium
rate and sodium carbonate and after dissolving in warm water and filtering,
filtrate was acidified with hydrochloric acid and boiled. On adding barium
oride, a faint but distinct white precipitate formed with the product from
inner ligament, whereas the reaction was found to be much weaker or
gative with the other layers (table 1). As will be shown later, there is evidence
t an amino-acid containing sulphur occurs in appreciable amounts in
drolysates of the inner ligament. However, since all regions of the shell
pear to be unaffected by an alkaline solution of sodium sulphide (table 1)
by thioglycollate solution (Goddard and Michaelis, 1934), it is likely that
n if disulphide bonds occur in the inner layer of the ligament, they are not
ncerned in the stabilizing of its protein structure (Brown, 1950*b*, 1952).

tilus edulis *and* Ostrea edulis

Similar tests were applied to the conchiolin in *Mytilus* and *Ostrea* and the
ults are summarized in tables 2 and 3 respectively. In many cases, the
ctions of the various layers of the shell are comparable with those described
their homologues in *Anodonta*. As shown by Brown (1952), the whole of
periostracum in *Mytilus* is intensely argentaffin and consists of a quinone-
ned protein. The properties of the external and middle layers, especially

their intense reactions to all tests for phenolic substances (table 2), sugges that they are both similar in composition to the periostracal conchiolin c *Anodonta*. The ability of the external layer to stain with Mallory is probabl due to it being less completely hardened at its origin than is the middle layer In contrast, the internal layer of the periostracum reacts only moderately t the Millon, xanthoproteic, and Folin (Baker) tests, and appears to differ mor fundamentally in composition from the conchiolin of the middle layer.

TABLE 2

A summary of the results of chemical and histochemical tests on the non-calcareou material in the shell of Mytilus edulis

Nomenclature as in table 1

	Valves				*Ligament*		
	Periostracum						
Test	*Ext. and middle layer*	*Int. layer*	*Outer layer*	*Inner layer*	*Perio-stracum*	*Outer layer*	*Inner layer*
HCl, conc., room temp., 8 h	All persist						
HCl, conc., 55° C, 8 h	Persists		Slowly dissolves		Persists	Quickly dissolves	
KOH, hot, sat.	Dissolves		Fragments persist		Dissolves		
10% sodium hypochlorite	All mostly dissolve						
Sodium sulphide	No apparent effect						
Millon	XXXXX	XX	X	X	XXXXX	X	? tr
Xanthoproteic	XXXXX	XXX	X	X	XXXXX	XX	? tr
Folin (Baker)	XXXXX	XX	tr	tr	XXXXX	X	O
Argentaffin	XXXXX	XXXXX	? tr	? tr	XXXXX	X	XX
Sakaguchi (Baker)	XXX	XX	XXX	XXX	XXX	XXX	tr
Sulphur	O		O	? tr	O	O	XXXX
Sudan black B	X	tr	tr	tr	O	XX	tr
Periodic acid / Schiff	? tr	X	tr	tr	O	O	O
Alcian blue	O	O	XXX	XXX	O	O	XX
Chitosan	O	O	O	+ve	O	O	O

The outer layers of the valves and ligament in *Ostrea* correspond closely t each other and to their counterparts in *Anodonta* in that they react moderatel to the argentaffin test and strongly to other tests for aromatic groups (table 1 and 3). This suggests that these layers are composed of a quinone-tanne protein probably similar to that in the outer shell conchiolin in *Anodonta* However, as in their staining reactions at controlled pH, each of these com ponents in *Ostrea* is specialized in particular ways. The conchiolin in th outer layer of the valves, for example, is distinguished by the fact that shows affinity for alcian blue and reacts positively to the chitosan test (table 3

TABLE 3

Summary of the results of chemical and histochemical tests on the non-calcareous material in the shell of Ostrea edulis

Nomenclature as in table 1

Test	*Valves*		*Ligament*	
	Outer layer	*Inner layer*	*Outer layer*	*Inner layer*
Cl, conc., room temp., 8 h	All persist			
Cl, conc., 55° C, 8 h.	Persists	Quickly dissolves	Persists	Quickly dissolves
OH, hot, sat.	Fragments persist		Dissolves	
% sodium hypochlorite	All mostly dissolve			
dium sulphide	No apparent effect			
illon	XXXX	XX	XXXXX	X
nthoproteic	XXXX	X	XXXXX	XX
lin (Baker)	XXXX	XX	XXXX	X
rgentaffin	XXX	tr	XXX	tr
kaguchi (Baker)	XXX	X	XXXX	XXX
ulphur	tr	O	X	XXXX
dan black B	XX	XX	XX	XX
riodic acid / Schiff	O	XX	O	? tr
cian blue	XX	XX	O	XX
hitosan (Campbell)	−ve	−ve	O	O

In *Mytilus*, as was to be expected, the non-calcareous component of the ıter layer of the valves was found to differ totally in character from that of ıe corresponding layer of the ligament (table 2). This is not, however, due ılely to the former being almost identical in composition with the matrix of ıe inner calcareous layer of the valves. The outer ligament layer is also nusual in that it dissolves rather easily in warm, concentrated hydrochloric cid, and gives a weak reaction to phenolic tests (table 2). Its protein content obviously considerably less hardened by tanning than that in the homo-gous layers of *Anodonta* and *Ostrea*.

In common with the corresponding regions in *Anodonta*, the inner shell yers in both *Mytilus* and *Ostrea* show only slight signs of tanning. Their istochemical properties indicate that they contain a relatively small pro-ortion of phenolic groupings (tables 2 and 3). In these features, the inner yers of the valves and ligament are readily differentiated from the outer shell yers in *Ostrea* and from the periostracum in *Mytilus*. Other notable points of omparison between homologous layers of the shells in different bivalves are ıat, as in *Anodonta*, the conchiolin of the inner ligament layer in *Mytilus* nd *Ostrea* is specialized in that it gives a positive reaction for sulphur, hile the inner layers of the valves all respond to the chitosan test for chitin ables 1–3).

The Composition of the Shell Protein

In order to examine the protein complements of the different shell layers in reater detail, each was analysed qualitatively by paper chromatography.

It was found that the outer and inner layers of the valves in *Mytilus* hav almost the same amino-acid composition, a feature which accounts for th similarity in their staining and histochemical reactions. Consequently, th amino-acid content of the inner layer only is described here and is contraste with that of the periostracum. In all other cases, the inner layer conchiol is compared with that occurring in the remaining shell layers. The latter a referred to as the 'outer regions' of the shell, this term replacing 'outer layer previously employed (Beedham, 1954). In addition to the true outer layer these regions incorporate periostracal material, and in the case of the lig ment of *Anodonta*, the fusion layer. It was not found possible to separa these layers sufficiently well to treat them as separate units.

After being purified by extraction with boiling ether, samples of conchioli from the different regions of the shell were hydrolysed in sealed tubes wit 6 N hydrochloric acid for 24 h at 100° C. Excess hydrochloric acid wa removed by evaporation and the hydrolysates were then taken up in 10% *iso*-propanol to produce in each case a final concentration of 10 mg of th original purified conchiolin per 1 ml solvent. The amino-acids were separate on Whatman no. 1 filter paper; the solvents used were aqueous phenol an butanol / acetic acid / water (4:1:5) (Block, Durrum, and Zweig, 1955 Estimates of the amounts of individual amino-acids in the different hydr lysates were made, usually on one-dimensional chromatograms, by visu assessment of the size and intensity of the spots, these being assigned a arbitrary value on the scale 0 to 11. Most of the estimates were carried out o chromatograms developed with ninhydrin, but in certain cases more specif reagents were employed. Arginine was determined by the α-naphthol / bromin test (Acher and Crocker, 1952) and proline by spraying with isatin in aceton (Block, Durrum, and Zweig, 1955). Jepson and Smith's (1953) technique fo the detection of hydroxyproline was also applied to the chromatograms, bu with negative results.

The estimates of 11 of the amino-acids detected in the hydrolysates ar summarized in fig. 3. Other amino-acids identified were histidine, lysin threonine, and valine. This analysis demonstrates conclusively that the pro tein in the inner layers of both valves and ligament differs markedly in com position from that in the remaining shell layers. Glycine, for example, abundant in most of the hydrolysates but it occurs in relatively higher con centration in those of the 'outer regions' of the shell and of the periostracu in *Mytilus* (fig. 3). This difference is particularly evident in the ligament, i which the glycine content of the inner layer is comparatively low. In contras the hydrolysates of the inner layers of the valves and ligament contain rela tively larger amounts of aspartic acid, glutamic acid (fig. 3), and lysine.

As observed earlier, an important feature for distinguishing between hydroly sates of different regions of the shell is their relative content of phenolic amino acids. Tyrosine and phenylalanine are much more abundant in the 'outer regions' of the valves and ligament and in the periostracum of *Mytilus* tha in the inner layers (fig. 3). In the case of tyrosine, this agrees with previou

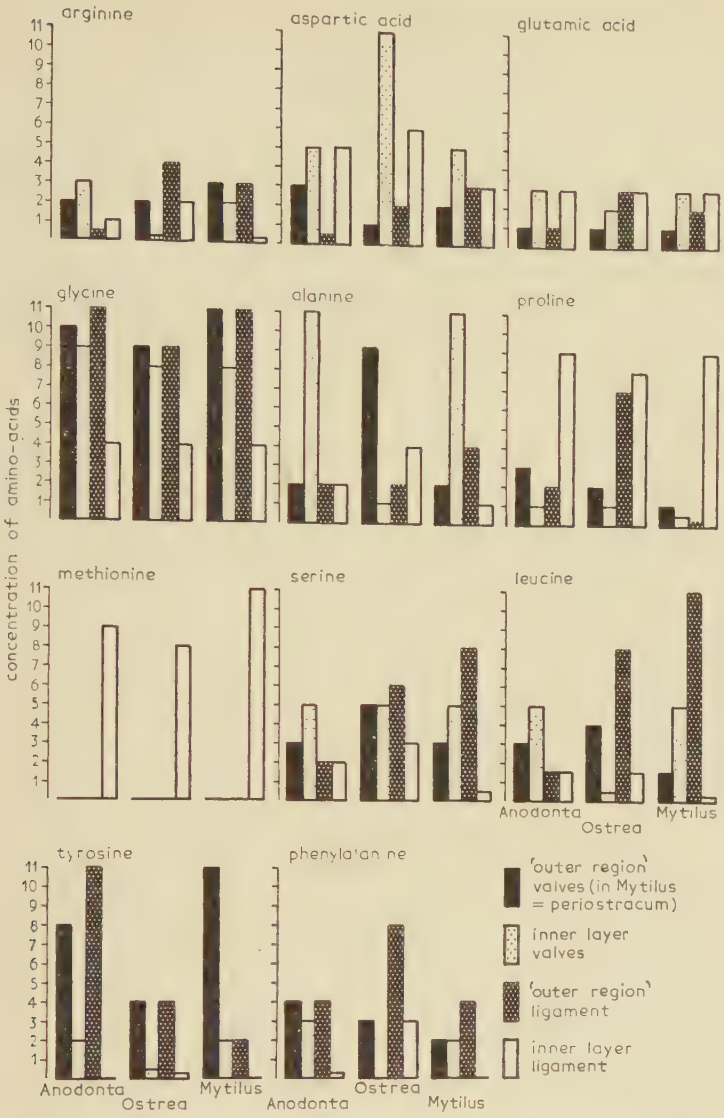


FIG. 3. Histograms showing the relative amounts of 11 amino-acids occurring in hydrolysates of the protein contents of the different shell layers in *Anodonta*, *Ostrea*, and *Mytilus*. Black represents the 'outer region' of the valves (except in *Mytilus* where it represents the periostracum); dense stippling the 'outer region' of the ligament. The inner layers of the valves and ligament are indicated by light stippling and white respectively.

analyses of the valve conchiolin in *Anodonta cygnea*, *Meleagrina margaritifera*, and species of *Pinna* (Friza, 1932; Roche, Ranson, and Eysseric-Lafon, 1951). These results can also readily be correlated with the histochemical reactions of the different shell layers to Millon's and other tests for aromatic groups. Unlike the corresponding regions in other shells, the 'outer region'

of the ligament in *Mytilus* contains relatively little tyrosine. In contrast, t exceptionally rich tyrosine content of the 'outer regions' of the shell *Anodonta* and of the periostracum in *Mytilus* suggests that this amino-ac plays an important role in the tanning of the conchiolin of these regions. this is so, the method of hardening of the periostracum in *Mytilus* may diff from that of the byssus, where, as shown by Brown (1952), the precursor the tanning agent is a phenolic protein or amino-acid, but not tyrosir Hydrolysates of byssal material were, in fact, found to contain only sm quantities of tyrosine compared with those of the periostracum. It may w be that the periostracal conchiolin in *Mytilus*, or more specifically that of t external and middle layers, is hardened in a manner similar to that suggest for the periostracum of *Anodonta*, with which it is closely comparable hist chemically.

In addition to showing that the 'outer regions' and inner layers of the sh contain two different types of protein, the present analysis confirms impressio gained from the observations on staining and histochemical reactions co cerning the relative composition of homologous layers of the shell. Whilst *Anodonta* the hydrolysates of the 'outer regions' of the valves and ligame have fundamentally the same composition, these components in *Ostre* although similar, show individual modifications (fig. 3). The former is disti guished by a high alanine content, whereas the latter is rich in proline. Als the 'outer region' of the ligament in *Ostrea* exhibits certain characteristi in common with its homologue in *Mytilus*, a feature which recalls the simil staining reactions of the outer ligament conchiolin in these species. T hydrolysates of both these regions contain appreciably larger amounts leucine and serine than those of the other components analysed (fig. 3).

The composition of the protein contents of the inner layers of the valv and ligament appear basically alike although each shows a certain amount specialization. That in the inner layer of the valves in *Ostrea*, for exampl contains a particularly large concentration of aspartic acid (fig. 3), a featu which seems to be consistent with the strong basiphil properties exhibited this layer. The inner valve layers in *Anodonta* and *Mytilus*, which are almo identical in composition, are both characterized by a high content of alanin On the other hand, the hydrolysates of the inner ligament, which sho striking similarity in amino-acid content in all the bivalves investigated, a distinguished from those of the corresponding region of the valves in th they are rich in proline and in the sulphur-containing amino-acid, methioni (fig. 3). The presence of an appreciable concentration of methionine is unusu since although this amino-acid is known to occur widely in proteins, it usual forms only a small percentage of the total amino-acids formed on hydroly (Fruton and Simmonds, 1953). The occurrence of methionine, which c be correlated with the positive sulphur reaction given by the inner layer the ligament (tables 1–3), was determined by its ability to reduce Feig sodium azide-iodine reagent, and by the identification of its derivativ methionine sulphone and methionine sulphoxide after oxidation with hydrog

peroxide (Block, Durrum, and Zweig, 1955). These reactions gave mainly negative results with all the other hydrolysates.

Discussion

In comparing the composition of homologous layers of the shell, the nature of the non-calcareous material in *Anodonta* is of particular interest. There is no doubt that, in this bivalve, the conchiolin of the outer layer of the ligament is essentially similar in its staining and histochemical reactions to that of the outer calcareous layer of the valves. In addition, the properties of the periostracum and fusion layer differ in degree rather than in kind from those exhibited by the outer layers. All these regions consist mainly of a quinone-tanned protein which contains a high proportion of phenolic residues, especially tyrosine, and which differs markedly in composition from the protein contents of the inner layers of both valves and ligament.

These properties may well be correlated with the characteristic zonation of the secretory epithelium of the mantle in *Anodonta cygnea* (Beedham, 1958). The epithelia on the outer surfaces of the outer mantle fold forming the outer layers of the valves and ligament are comparable histologically and histochemically both with each other and with those on the inner periostracal-secreting surface of the outer fold and the outer surface of the fused outer folds which secretes fusion layer. All these epithelial zones are readily distinguishable from the epithelia concerned with the deposition of the inner shell layers. In *Anodonta* the outer mantle fold (i.e. both inner and outer surfaces) is to be regarded, therefore, as a complete secretory unit whose products differ in composition from those formed by the remainder of the outer surface of the mantle.

The situation in *Anodonta* provides confirmation of the view that corresponding layers of the valves and ligament are basically identical and are, in fact, locally modified regions of the same layers of the shell (Owen, Trueman, and Yonge, 1953). In the other bivalves investigated, the non-calcareous components of the outer layers of the valves and ligament undergo more extensive modifications, the degree of specialization being relatively slight in *Ostrea* but extremely pronounced in *Mytilus*. It should be pointed out, however, that the conchiolin of the major part of the periostracum in *Mytilus* appears to have a similar constitution to that of its homologue in *Anodonta*.

Whereas the conchiolin of the periostracum or of the outer layers of the valves and ligament is hardened and stabilized by quinone-tanning to form an efficient protective cover over most of the external surface of the shell, the non-calcareous matrix of the inner shell layers contains a low proportion of aromatic groupings and is relatively lightly tanned. Grégoire, Duchâteau, and Florkin (1955) have demonstrated that only a part of the protein in the inner calcareous layer of the valves in *Pinctada* (*Meleagrina*) *margaritifera* consists of a scleroprotein, and that there is in addition a protein soluble in water and a polypeptide.

In these and other features the inner layers of the valves and ligament are

fundamentally the same, which fully supports similar conclusions based on morphological evidence (Owen, Trueman, and Yonge, 1953). However, not unexpectedly, the protein content of each layer is found to undergo some modification. The high proline content of the protein of the inner ligament layer, coupled with the fact that it contains a very low percentage of phenolic amino-acids, suggests that it is somewhat similar in composition to collagen (see also Trueman, 1949). However, it differs from collagenous proteins in that it contains appreciable quantities of methionine, a relatively small proportion of glycine, and little or no hydroxyproline. Since all these characteristics invariably appear in each of the species investigated, it is probable that the modifications exhibited by the protein in the inner ligament are correlated with the highly specialized function of this region of the shell. Unlike the remainder of the shell, the inner layer of the ligament is constantly subjected to compressional stresses due to the closing action of the valves.

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ome Aspects of Cuticular Organization of the Branchiopod, *Streptocephalus dichotomus*

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Summary

The structural features of the cuticle of *Streptocephalus* are described. The epiticle and endocuticle, unlike those of other Crustacea, stain alike and show similar emical characters (apart from the absence of chitin in the epicuticle). This feature pears to be related to the absence of tanning of the cuticle, which remains soft and lourless in all stages of growth. In the epicuticle the outermost very thin layer, whose esence can be inferred from chemical tests, seems to correspond to the outer epiticle of Crustacea, but this layer lacks histological distinctness. The protein contuent of the cuticle is unlike that of other arthropod cuticles so far studied. It differs arkedly from arthropodin or sclerotin and recalls collagen. The significance of the culiarities of the cuticle is discussed. It is suggested that the structure and chemical aracteristics of the cuticle may indicate that it represents a generalized and specialized condition of the arthropod cuticle.

Introduction

RECENT studies indicate a non-uniformity in the chemical composition of the cuticle of arthropods. In *Palamneus*, *Scolopendra*, and *Propallene* has been noted that the amino-acid composition of the protein constituent the epicuticle differs from that of its counterpart in insects in containing stine residues, which are absent in the latter (Krishnan, 1953, 1954, 1955, 956). The presence of such sulphur-containing amino-acids appears to be lated to the mode of hardening of the cuticle, which involves the formation —S—S-bonds as in the keratin of vertebrates, as opposed to the condition ported in the cuticle of insects, which is hardened by phenolic tanning. is becoming increasingly clear that the cuticle of arthropods, far from conrming to any one chemical pattern, may show a range of variation. The gnificance, if any, of such variations merits further study. In this connexion is of interest to recall the work of Reed and Rudall (1948), who called attenon to the problem of the relationship of the arthropod cuticle to that of nnelids. The two cuticles are very different, that of annelids being composed a protein resembling collagen, whereas the so-called arthropodin is of eratinous β-protein type (Astbury, 1945). In the light of the above observaons the finding, in the cuticle of a primitive branchiopod crustacean, of a otein hitherto undescribed, seemed to warrant a more intensive study of its ructural and chemical constitution.

Although the cuticle of decapod Crustacea has been studied in considerable

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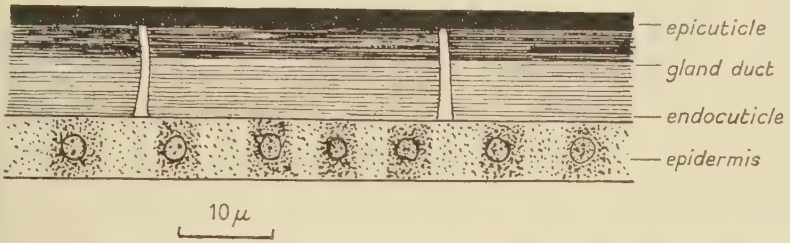
detail (Drach, 1939; Dennell, 1947; Krishnan, 1951; Richards, 1951), it not known whether the characteristics of the cuticle found in them are general validity for the entire group. From what little is known of the cuti of entomostracan Crustacea (Lafon, 1941, 1943; Thomas, 1944; Richards a Cutkomp, 1946), it would appear that chitin is present in the cuticle of rep sentative species of Branchiopoda, Cladocera, Copepoda, and Cirreped Lafon (1943), from a biochemical analysis of the cuticle of a number of typ reported the relative percentages of chitin and protein. Thomas (1944), the course of his studies on the tegumental glands of the cirripedes such *Lepas*, *Balanus*, and *Lithotrya* found that the cuticular organization in the species recalls that of decapods like *Homarus* in consisting of a very th non-chitinous, protein layer overlying a broad endocuticle formed of a chiti protein complex. The information regarding the other classes of Entomostra is more scanty. Dennell (1947), from his observations on the cuticle of *Ap cancriformis*, a branchiopod, suggested that it may be similar to that of t other Crustacea, but he noted that it was too thin and delicate to preser even the body shape. It may be inferred that the cuticle in this species unhardened, but no information is available of its structural characteristi and chemical composition beyond the fact that both chitin and protein a present. Lafon (1943) has not indicated the protein value for the branchiop *Triops*, although he recorded that chitin amounts to about 61·4%. From t above brief review of our knowledge of the cuticle of Entomostraca, especia Branchiopoda, it would be clear that there is need for information regardi the structure of the cuticle, the mode of hardening, if any, and the natu of the chemical components of the cuticle. It is realized that protein is by f the most important constituent of the cuticle, which forms as it were t central unit around which the other cuticular components are built and whi largely accounts for the properties of the cuticle. In the following study attempt has therefore been made to investigate the structure and chemic composition with special reference to the protein constituents of the cutic of *Streptocephalus* by histological, histochemical, and microchemical pr cedures. The object has been to arrive at a more complete understanding the cuticular organization of a hitherto less known group of Crustacea.

Structure and Staining Reactions

Streptocephalus dichotomus is an anostracan branchiopod, characterized the absence of a shell-fold. It is of common occurrence in fresh-water pon in Madras and its environs. The body, composed of 19 segments behind t head region, presents an almost vermiform appearance. The average leng of an adult varies from about 5 cm to about 8 cm, the females being small than the males. The general body surface is covered by a very thin cutic closely adhering to the epidermis. By careful manipulation the cuticle can separated from the underlying soft parts. It is colourless, soft, and flexib and remains so at all stages of growth. Sheets of cuticle taken from the thor

abdomen show in a surface view minute polygonal patterns, marked by ll-defined lines. This has been noted in a number of Crustacea (Drach, 39). In *Cancer pagurus* Dennell (1947) distinguished two distinct patterns. one of these the boundary lines are wavy or corrugated; this is found in the wly-formed cuticle of an animal that has just moulted. In the other type, n in cuticles some time after the moult, the boundaries are marked by rtical lines. The significance of the corrugated lines is said to be that they ovide for expansion during the rapid growth of the cuticle after the moult. has been pointed out that these patterns may indicate the mode of formation the cuticle by the activity of the underlying cells (see Dennell, 1947).



G. 1. Transverse section through the tergite cuticle of *Streptocephalus*, stained by Mallory's method.

Sections passing transversely through the thorax and abdomen show the ticle as a very thin, colourless layer about 4 μ thick, overlying the epidermis g. 1). The cuticle is of more or less uniform thickness over the tergites and ernites. Laterally the cuticle is thinner. In unstained sections of the cuticle, outer membrane less than 1 μ thick is distinguishable from the inner region the cuticle. This layer shows staining reactions and chemical characteristics fferent from the rest of the cuticle, and, as will be shown in the sequel, rresponds to the epicuticle of other arthropods, while the inner, broader, lourless region is the procuticle or endocuticle, which shows faint horizontal nellations extending up to the epicuticle. A division is discernible in the docuticle, marking an outer and inner endocuticle. In the former the nellations are closer and less prominently seen than in the latter, in which ey are wider apart and conspicuous. Gland ducts traversing the cuticle from e epidermis to the outer surface are sparsely distributed. Pore-canals could t be made out in unstained preparations.

The cuticle presents more or less similar features in all regions of the body rface. It is not amber-coloured. In insects and most other arthropods the pearance of an exocuticle brings about a modification of the basic structure the cuticle, resulting in a differentiation of the sclerite cuticle from the hrodial membrane. The transition between the two being gradual, a region presenting an intermediate condition designated the 'intermediate sclerite ticle' has been distinguished. In such forms, the arthrodial membrane nsists only of the epicuticle and endocuticle. In *Streptocephalus* the cuticle roughout the body surface, apart from minor modifications, is soft and

consists only of the epicuticle and endocuticle, so that there can be no di tinction into the arthrodial membrane condition and the sclerite conditi noted in other arthropods (see Blower, 1951; Dennell and Malek, 1955*a*).

The above-mentioned differences between the cuticle of *Streptocepha* and that of other arthropods are further emphasized by a study of the staini reactions. With Mallory's triple stain, the epicuticle takes on a deep bl colour and the endocuticle a lighter blue. In the latter, the inner region stai more faintly than the outer. With Masson's trichrome stain the epicuti stains a dark green colour, while the endocuticle is only lightly stained gree the innermost region being more feebly stained. The endocuticle is not stain with Heidenhain's haematoxylin but the epicuticle becomes deep brown blue. In the lateral regions connecting the tergite and sternite the cuticle markedly attenuated, the endocuticle being reduced; the epicuticle is indicat by its affinity for haematoxylin. These staining reactions differ from tho reported for the cuticles of decapod Crustacea. In the latter, even in t freshly-moulted condition, the epicuticle stains characteristically red a the endocuticle blue with Mallory. With the onset of phenolic tanning aft the moult, the epicuticle gradually loses its ability to stain and turns ambe coloured, while the presumptive exocuticle tends to stain red like the u hardened epicuticle; this is followed by the formation of an amber-colour exocuticle and loss of ability to stain (Dennell, 1947; Krishnan, 1951, 195 It would appear, therefore, that the cuticle of *Streptocephalus* is not compa able to that of a new-moulted decapod crustacean, although in being u hardened and soft the two resemble one another. Dennell and Malek (1955 in the course of their studies of the cuticle of the cockroach observed th although the cuticle that has just moulted bears a strong resemblance to t arthrodial membrane, the unhardened cuticle of the tergites differs in so respects from this. The differences lie in the staining reactions, which indica the presence of lipoprotein in the epicuticle of the tergites. This lipoprote stains red with Mallory. In the soft arthrodial membrane, on the contra the epicuticle and endocuticle stain alike blue with Mallory and green wi Masson's trichrome stain. The staining reactions of the cuticle of the arthrod membrane and of the tergite of *Streptocephalus* may suggest the absence fr both of a protein capable of being tanned. It is found, however, that the e cuticle —unlike that of the arthrodial membrane—stains with haematoxyl The observations of Dennell and Malek (1955*b*) may indicate that the su stance that stains with haematoxylin in the insect cuticle is a protein rich tyrosine, which is later involved in tanning. In a cuticle such as that of *Strep cephalus* the substance that stains with haematoxylin cannot be identical w that in cockroach cuticle. It would be reasonable to assume that in arthrop cuticles, haematoxylin staining may be due to different substances. F example, in the arachnid *Palamneus*, the epicuticle of the arthrodial membra stains with haematoxylin although it does not undergo tanning, and the pr tein constituent is very different from the protein rich in tyrosine that characteristic of the insect epicuticle (Krishnan, 1954).

Chemical Composition

stochemical tests performed on both fresh and frozen sections lend ort to the suggestion made above that there is a marked similarity in the osition of the tergite cuticle of *Streptocephalus* and that of the arthrodial brane of insects (table 1).

Table 1

The responses of the tergite cuticle of Streptocephalus *to chemical tests*

Test	*Epicuticle*	*Endocuticle*	
		Outer endocuticle	*Inner endocuticle*
n's	−	−	−
oproteic	−	−	−
t	+	+	+
taffin	++	+	−
n black B	++	+	−
er's	−	−	−
chloride	−	−	−
san	−	+	+
ntrated HNO_3 (cold)	dissolves slowly	dissolves rapidly	dissolves rapidly
ntrated HCl (cold)	,, ,,	,, ,,	,, ,,
ntrated KOH	dissolves	no apparent effect	no apparent effect

ne negative Millon and xanthoproteic tests indicate the absence from both picuticle and the endocuticle of a protein containing phenolic substances. the protein constituent of both the epicuticle and endocuticle is similar be inferred from the above-mentioned reactions and the positive biuret ions in both the regions. These reactions, together with the negative ner's test, strongly recall those reported for the arthrodial membrane le of *Periplaneta*; but here, though not in *Periplaneta*, the Sudan black s positive in the epicuticle and in the outer regions of the endocuticle. ture of interest is that those regions giving a positive reaction with Sudan are also positive to the argentaffin test. In both these reactions the ticle is more intensely positive than the endocuticular regions. Dennell Malek (1955*a*) have discussed at length the significance of a positive taffin reaction in the cuticles of insects and pointed out that by itself sitive reaction is indicative of no more than the presence of reducing tances. In the cockroach cuticle the positive argentaffin reaction taken other histochemical tests has been shown to indicate a protein rich in ine; this protein is present in those regions of the cuticle which undergo ng. In contrast it has been shown that the cuticle of the arthrodial brane shows a negative reaction to the argentaffin test. In the absence of ng at any stage in the cuticle of *Streptocephalus*, it is obvious that the ive argentaffin reaction noted here is indicative of a reducing substance than tyrosine or a protein containing tyrosine. The negative ferric ide test rules out the possibility of phenols being present in the cuticle.

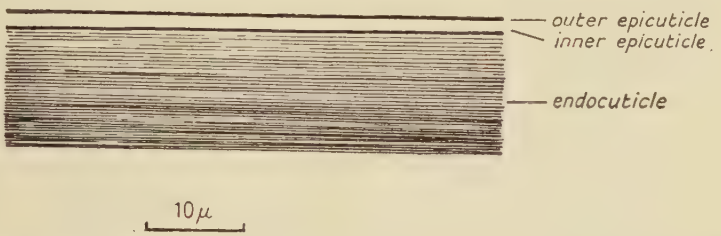
The close correspondence between the sudanophil regions of the cuti and those giving a positive argentaffin reaction may suggest that the arg taffin-positive substance in the cuticle of *Streptocephalus* may be a lipid, it is known that lipids may react positively to ammoniacal silver nitrate.

Notwithstanding the differences noted above, the chemical composit of the cuticle suggests a similarity to the cuticle of the arthrodial membra of an insect like *Periplaneta*. The histochemical tests for proteins give simi reactions in both the cuticles, which suggests that a similar type of prot forms a basal matrix that is not impregnated at any stage by another prote The absence of an amber coloration is common to both. Susceptibility to action of mineral acids and in general a lack of chemical stability are ot features common to the cuticle of the arthrodial membrane of insects a that of *Streptocephalus*. Although a lipid is said to be wanting in the epicuti of the arthrodial membrane of the cockroach, its reported occurrence in cuticulin layer from the very inception of its development in insects l *Calliphora* suggest the possibility of the presence of a lipid constituent in arthrodial membrane of insects, as in the cuticle of *Streptocephalus*.

Nature of the Epicuticle

From the results of the histochemical tests, it may be inferred that epicuticle of *Streptocephalus* corresponds to the epicuticular layer of the cuti of the arthrodial membrane of insects. Histological examination did not rev the presence of an outer epicuticle as in the decapod Crustacea. Secti stained with Mallory's and Masson's trichrome stains show the epicuti as an apparently single layer. Although with Sudan black the outerm portion of the epicuticle becomes darker, the colouring is diffuse, so that i not possible to distinguish a separate outer layer on the basis of the sudar phil reaction. It has been observed that concentrated hydrochloric a rapidly dissolves the endocuticle, but the epicuticle is only slowly attack On prolonged treatment, however, the greater part of the epicuticle is d rupted, leaving a very thin outermost membrane which resists for some ti the action of cold hydrochloric acid. Pieces of entire cuticle were kept concentrated hydrochloric acid for periods varying from 1 hour to 2 da and later embedded in paraffin and sectioned. A comparative study of cuticle after having been kept in hydrochloric acid for varying periods, sho that the most resistant part of the cuticle is the outermost very thin layer the epicuticle. Material kept in concentrated hydrochloric acid for about a and later sectioned and stained with Heidenhain's haematoxylin shows in epicuticle a marked distinction into an outermost very thin membrane stain more intensely with haematoxylin and a wider inner part which is very ligh stained and shows signs of disruption (fig. 2) when sections of the sa material are treated with Sudan black. The outermost layer referred to ab takes up a black coloration while the inner part is not or only feebly reacti The presence of a membrane answering to the description given above n be made out in some preparations of the cuticle subjected to a less viol

atment than is involved in the chitosan test, as for example when the terial is treated with concentrated potash for about half an hour at 100° C d later sectioned. The membrane in question is clearly seen in relation to e endocuticle and the surviving parts of the epicuticle, of which it forms e outer rim. When stained with Heidenhain's haematoxylin, it was coloured ack, whereas the endocuticle hardly took up the colouring agent. Similarly, nen sections of the cuticle are treated with cold chlorated nitric acid, the ssolution of the cuticle is rapid, starting with the endocuticle and extending



G. 2. Transverse section of the tergite cuticle of *Streptocephalus* after treatment with concentrated hydrochloric acid, stained with iron haematoxylin.

utwards. After some time the entire section of the cuticle breaks down, but ie portion which survives to the last is the very thin outermost membrane the epicuticle, corresponding to that noted after maceration with concen- ated hydrochloric acid. The sequence of disruptive changes correspond with ose reported for the epicuticle of the cockroach (Dennell and Malek, 955*a*), in which a thin membrane that survives to the last has been thought to e homologous with the outer epicuticle or the outer resistant part of the uticulin layer of insects.

In the adult epicuticle of *Palamneus* an outermost, thin, resistant layer is istinguished as the outer epicuticle. Histochemical and X-ray diffraction udies show that it contains long-chain paraffins (Krishnan, 1954). The nemical features of the outermost part of the epicuticle of *Streptocephalus* call in some respects the outer epicuticle of *Palamneus*.

It will be seen from the foregoing account that the cuticle conforms to hat may be called the basic structure of the arthropod cuticle in comprising vo fundamental layers, the epicuticle and endocuticle, of which the epicuticle ves evidence of being constituted of two parts, an outermost, very thin, yer containing lipid, and an inner lipoprotein part. In the absence of harden- g by tanning or by —S—S-bonding it differs from that of other arthropods ut recalls strongly the arthrodial membrane condition of the adult cuticle such an insect as *Periplaneta*.

Cuticular Protein

The peculiarities of the cuticle of *Streptocephalus*, especially its chemical mposition, are attributable to the nature of the cuticular protein. Although some respects there is a similarity to the protein of the arthrodial membrane

cuticle of *Periplaneta*, a direct comparison with it is precluded by a pauc of information on the nature of this protein in the insect. It is, however, cl from the results reported by Dennell and Malek (1955*a*) that the prot constituent of the arthrodial membrane is different from arthropodin, wh is characterized by a high tyrosine content and presumably stains red w Mallory. A similar inference may be made regarding the protein of the terg cuticle of *Streptocephalus*. In table 2 are given some of the results of the te performed on the cuticle which support the above suggestions.

TABLE 2

The responses of the tergite cuticle of Streptocephalus *to further chemical tests*

Test	*Result*
pepsin+HCl	swells and disrupts
saturated ammonium sulphate	slowly disintegrates
trichloracetic acid 10% (cold)	swells and disrupts
boiling water	swells
H_2SO_4 (dilute)	swells and disrupts
lead acetate test	negative
sodium nitroprusside test	negative

The above results suggest that the cuticular protein of *Streptocephalus* h none of the characteristics described for the sulphur-containing protein the cuticle of *Palamneus* (Krishnan, 1953, 1954), which gives positive colo reactions with the sodium nitroprusside and lead acetate tests, and sho marked swelling effect on treatment with alkaline sodium sulphide. Nor do this protein accord with the arthropodin of the soft cuticle of insects, which characterized by its insolubility in cold 10% trichloracetic acid and its failu to coagulate in hot water. On the other hand the reactions to boiling water, dissolution in pepsin, and saturated ammonium sulphate, as well as in co 10% trichloracetic acid, while distinguishing it from arthropodin or sclerot suggest a collagenous type of protein.

To test the validity of the suggestion made above a microchemical analy of the cuticle was made by a modification of the method of Spencer, Morgu and Wilder (1937). These authors applied the above method successfully a microdetermination of the collagen content of the muscles of the rabbit.

The cuticle was separated by cutting open the body longitudinally; t contents were removed. The cuticle was cleaned by scraping with a blu scalpel in a washing medium of 60% alcohol, so as to remove as complet as possible all adhering tissue. The cuticular material was cut into small b and a 100 mg sample was homogenized with 1 ml of distilled water in a Pott Elvehjam homogenizer. Drying the material with acetone was omitted (Harkness, 1952). The homogenized cuticle was placed in a boiling wa bath for 10 to 15 min with 5 to 10 times its weight of water and later stored a refrigerator. Next day it was autoclaved for 3 h at 20 lb pressure. By au

aving, the collagen in the cuticle (if any) would be converted into gelatin. he material was then centrifuged at 4,000 rev/min for 1 h and the supernatant uid drawn off and treated with 5% tannic acid, as in the original method. y this treatment a precipitate was obtained which indicates the presence of elatin derived from the cuticle (see Spencer and others, 1937).

To test further the nature of the precipitate, it as subjected to a chromatographic analysis. The aterial was treated with 10 times its weight of NHCl in a sealed tube and hydrolysed at 105° C r 24 h. After hydrolysis the solution was trans-rred to a small beaker and dried in a vacuum esiccator containing potassium hydroxide. When rying was complete the material was used for n analysis of its amino-acid constituents by paper artition chromatography. The technique followed as the capillary ascent method of Williams and Kirby (1948). The hydrolysate was dissolved in a mall quantity of distilled water and a 20 μl sample as applied to a sheet of Whatman no. 1 filter aper. The chromatogram was run with butanol cetic acid water as the solvent. Simultaneously a umber of chromatograms were run under identi-al conditions with solutions of pure amino-acids or purposes of comparison. The amino-acids vere each prepared by dissolving 5 mg in 5 ml of listilled water. The chromatograms were exam-ned after spraying 0·1% ninhydrin in butanol. For one set of chromatograms the spraying agent as prepared as above and to this a few drops of :4:6 collidine were added, which is said to im-prove the sensitiveness of the reagent. It was found hat by this means, the amino-acid spots on the hromatograms were more sharply defined. An stimation of the amino-acids present in the ydrolysate was made by the application of Rf alues of amino-acids as well as by comparison vith chromatograms obtained with pure amino-acids.

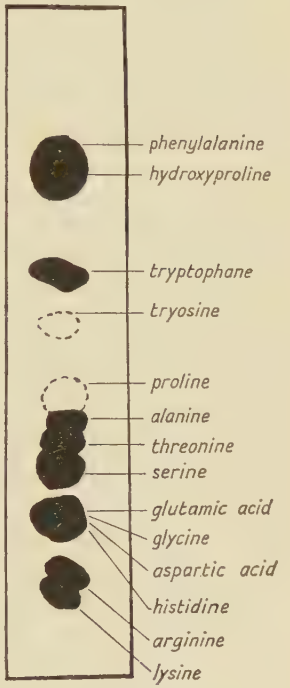


FIG. 3. Diagram of a paper chromatogram of the amino-acids in an acid hydrolysate of cuticular material of *Strepto-cephalus*, estimated by the method of Spencer and others (1937). Dotted lines represent spots which are visible in the original but did not appear in the photographic reproduction.

The amino-acids in an acid hydrolysate of the protein of the cuticle of *Streptocephalus* include most of those obtained from the collagen of ox-hide Bowes and Kenten, 1949), that is, alanine, arginine, asparatic acid, glutamic cid, glycine, histidine, hydroxyproline, lysine, phenylalanine, proline, serine, hreonine, and tyrosine (see fig. 3). However, the cuticular protein is lacking n cysteic acid, hydroxylysine, leucine, and valine, present in the collagen from x-hide, while it includes tryptophane, which is absent from the latter. It has een observed that the same type of protein drawn from different sources

may vary in some respects in regard to amino-acid composition (Hackm 1953). In view of the above observations, and considering the number amino-acids common to the cuticle protein and collagen, it would app reasonable to suggest the essential identity of the cuticular protein a collagen.

As a further test, the chromatogram obtained from cuticle protein w compared with that of pure gelatin, prepared for chromatographic analysis a manner identical with that applied to the material of the cuticle that precipitable by tannic acid. There was a marked resemblance between t amino-acids of the gelatin and those of the cuticle.

Discussion

The results reported above show that the cuticle of *Streptocephalus* prese certain peculiar features in regard to its structure and chemical compositi The epicuticle, though conforming in general to the type found in oth Crustacea, lacks the differentiation into sub-layers corresponding to t outer and inner epicuticle of decapods and cirripedes. The staining reactio suggest an apparent homogeneity, for the outermost resistant part, di tinguished by chemical tests, was not evident on histological examinati Another noteworthy feature is the marked similarity in the chemical chara teristics of the epicuticle and endocuticle, apart from the distinction based the absence of chitin in the former. These two layers stain alike with Mallor and Masson's trichrome stain and show similar reactions to histochemi tests for proteins and lipids, although quantitative differences exist. It known that in decapod Crustacea as well as in cirripedes the two fundamen layers of the cuticle are distinguished by their different chemical compositi Apart from the absence of chitin, the epicuticle in all Crustacea so far studi is characterized by the presence of a protein rich in phenolic substances, whi is either absent in the endocuticle or appears at a later stage in the growth the cuticle and then distinguishes the presumptive exocuticle. The abse of such a distinction cannot be explained as due solely to the non-occurrence tanning. This does not by itself provide an explanation, for there are a numb of instances among the decapod Crustacea in which, even where tanning d not occur, the protein in question is still present in the epicuticle, as example in the soft cuticle lining the gut of *Homarus* and the tergite cuticle *Penaeus* (Yonge, 1932; Krishnan, 1956). It would appear that the prot constitution of the cuticle in *Streptocephalus* is such that it lacks the mechani necessary for undergoing tanning and is destined to remain permanently a soft and flexible state, comparable in some respects to the arthrod membrane of the insect cuticle. The significance of such a feature may that the cuticle in this type is in an unspecialized condition.

The above suggestion receives further support from a consideration of nature of the cuticular protein. Until recently few attempts have been m to characterize the proteins of the arthropod cuticle. The observations Trim (1941) on the cuticular protein of two insects, *Sarcophaga falculata* a

hinx ligustri, show that it is different from collagen and in some respects
embles sericin. Fraenkel and Rudall (1947), while confirming in general the
servations of Trim, emphasized the unique features of this protein, which
ade it difficult to relate it to any known protein. They felt the necessity of
signating it by a new name, arthropodin. Among the features distinguishing
hropodin may be mentioned its occurrence always in a β or extended pattern,
high tyrosine and low glycine content, solubility in hot but not cold 10%
chloracetic acid, and its failure to coagulate in hot water. When hardened
tanning, arthropodin is said to give rise to sclerotin, which shows a similar
ino-acid composition. Later, Blower (1951) in his studies on the cuticle of
yriopods noted at least two different protein components during the growth-
ase of the cuticle before tanning took place, the one staining blue with
allory and found in the inner regions of the endocuticle that never undergo
nning, the other appearing shortly before tanning and connected with that
ocess. On account of this feature Blower designated it prosclerotin. A pro-
in identical in staining and chemical characteristics with the so-called pro-
lerotin is found in insects; it has been described in detail by Dennell and
Ialek in *Periplaneta*, where it is shown as impregnating a basal protein
atrix of the epicuticle and the presumptive exocuticle. In staining reactions,
nino-acid composition, and histochemical reactions this protein is indis-
nguishable from arthropodin. The identity of the tyrosine-rich protein of
e cockroach cuticle with arthropodin has also been indicated by Dennell
d Malek (1955*b*). It may be suggested that the tyrosine-rich, fuschinophil
otein, reported from the cuticles of various Crustacea, is identical with the
ove-mentioned protein in cockroach cuticle. From the wide occurrence of
is type of protein in arthropods it was assumed that it is characteristic of
e arthropod cuticles. However, the observations on the epicuticular protein
some arachnids and myriopods (Krishnan, 1954, 1956) have not confirmed
ch a view. In them the principal protein component of the epicuticle is
fferent from its counterpart in insects in containing organic sulphur and in
ing hardened by a process similar to keratinization. Very little is known of
s chemical nature except that it is rich in cystine residues and that in spite
a superficial resemblance, it is not identical with vertebrate keratin as
vealed by its X-ray diffraction pattern. As in the arachnids referred to above,
e cuticular protein of *Streptocephalus* is unlike arthropodin or its derivative
lerotin. An analysis of the protein suggests a marked likeness to collagen.
he nature of the protein forming the basal matrix of the cuticle of the
throdial membrane of insects or that forming the protein constituent of the
nhardened regions of the endocuticle in insects and decapod Crustacea is
ot known. Hitherto the homogeneity of the cuticular protein has been
sumed, and recently Hackman (1953) called attention to the possibility of
e occurrence of a number of protein components constituting the total
otein content of the cuticle. The above author found from an electrophoretic
amination of the cuticular protein of the insect *Diaphonia dorsalis* that it is
rmed of a number of components. The fastest anodic component showed an

amino-acid composition different from that of the entire protein preparat of the cuticle in not containing serine, threonine, phenylalanine, proline, hydroxyproline. It seems clear that in the cuticle of arthropods we have deal with several protein fractions showing different amino-acid compositi This feature may account for the apparently anomalous results obtained fr an analysis of the total protein content of the cuticle.

In the foregoing study of the cuticle of *Streptocephalus* a type of protein h been isolated which is shown by chemical tests to be allied to collagen. amino-acid analysis also indicates a pattern closely resembling that of collag The occurrence of a collagenous protein has not till now been reported fr the cuticles of arthropods. Till very recently it has been assumed that th is only a single protein component in the cuticle and that this is a keratin β protein, so that the other protein components have been ignored. Even the insects which have received much attention in this respect, very lit is known of the protein fractions of the cuticle other than arthropodin or derivative sclerotin. For example, no information is available regarding t nature of the protein component of the cuticle of the arthrodial membrane insects, which is known to differ in staining and histochemical reactions fr those of arthropodin. It is of interest to point out that in the above-mention features, it resembles to some extent the cuticular protein of *Streptocephal* But till more extended observations are made regarding the nature of t protein of the arthrodial membrane cuticle, a valid comparison with it is n possible. An interesting point for consideration is the possible significan of the occurrence of collagen in *Streptocephalus*, for this is a primiti crustacean type, showing many features in its anatomy reminiscent of anne organization. In the light of these facts, the presence in its cuticle of a prot identical with that found in the annelid cuticle may be considered significa

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The Co-ordination of Growth in the Tracheal System of Insects

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With one plate (fig. 8)

Summary

he tracheae in *Rhodnius* and many other insects are parallel-sided tubes which nch in such a way that the cross-section of the diffusion path remains constant. owth in length results mainly from stretching the trachea in the preceding instar. owth in diameter of the tracheae varies with feeding and the organ supplied. The portions of the tracheal system are maintained by co-ordination of the growth of the n tracheae and new terminal branches. This is brought about by the property racheae which enables them to react to stimuli for growth only upon the spiracle e of the point of application. The stimuli for growth probably originate from the ues and from the nodes. They determine first the diameter of the tracheae farthest ay; that is, the tracheae begin to form at the spiracles. The nodes controlling the meter of the lateral tracheae are influenced by the blood.

Growth in the tracheal system of *Tenebrio* larvae varies inversely with the oxygen sion both above and below atmospheric.

Introduction and Preliminary Observations

THE tracheal system in an insect consists of extensible cuticular tubes, the tracheae, arising from segmentally arranged spiracles. The tracheae anch repeatedly with reduction in diameter and end in the tracheoles, fine racellular tubes penetrating the tissues of the body. The segmental tracheae ay be united by lateral tracheae running in the length of the animal. The ttern of branching in the abdominal tergites of *Rhodnius* is shown in fig. 1. large insects, particularly those with respiratory movements, the tracheae ay be spindle-shaped sacs, oval in cross-section, but in small insects and in minal ramifications the tracheae are parallel-sided tubes, circular in cross-ction. This enabled Krogh (1920) to calculate the cross-sectional area from easurements of the diameter of tracheae at successive levels of branching om the lateral tracheae to the tissues in a *Tenebrio* larva. He found that the m of the areas of the cross-sections of the branches was equal to the cross-ctional area of the main trachea. Thorpe and Crisp (1947) found the same ationship for three tracheae and their branches in adult *Aphelocheirus*, and sumed it to be true for their calculations on the respiratory efficiency of the t of the animal. It is remarkable that this simple description of branching what is otherwise a most complex system should have received so little ention.

The diameters of the tributary tracheae and the main branch were measured

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in a number of forks in whole mounts of several insects. The cross-section area of a trachea was found to equal that of its branches in all the tubes circular cross-section examined. Table 1 gives some of these results.

There are exceptions to this simple tree-like branching pattern as in th four-way junctions between the lateral and segmental tracheae, or the plexus

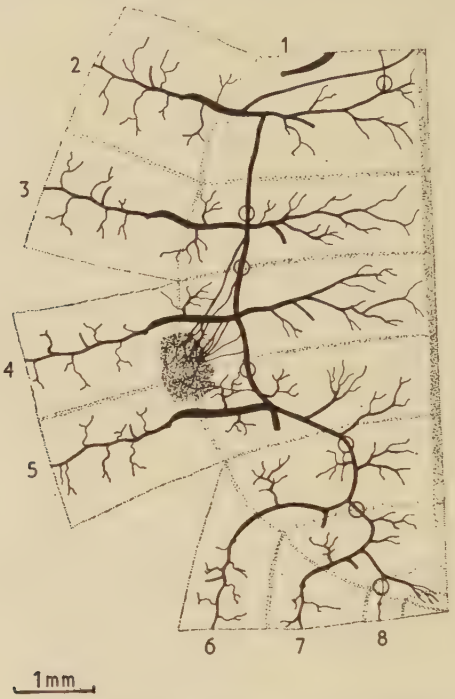


FIG. 1. Diagram of the tracheal system of one side of the abdomen in a 5th instar *Rhodni* larva, as it usually appears in the preparations described in the text. The larva has been di sected from the ventral surface and the tracheae to the viscera have been cut short. The stipple mass indicates the position of a testis. The tracheae to the tergites are separated on the tw sides by the heart but the ventral tracheae unite in nodes. The usual position of the nodes o the lateral tracheae are indicated by small circles. The numbers refer to the appropria abdominal spiracle.

of tracheae occurring in some insects (e.g. *Dixippus*), but it seems probab that the relation between the branches described above is a general on Teleologically this is not unexpected if it is assumed that the distance fro tissue to spiracle is more or less constant along any one branching syste The rate of diffusion along a tube is proportional to its cross-section. If th total cross-section of the branches grew greater as they divided the cros section of the main trunk would still remain limiting. If the converse we true the terminal branches would limit diffusion. The latter arrangeme might be expected if much oxygen had been lost through the main tru walls before reaching the tissues.

Tracheal branching in insects may be compared with that in a chilopod, *ithobius*, in which the cross-section diminishes significantly as branching roceeds (the sum of the cross-sectional areas of the branches divided by the ross-sectional area of the main trunk = 0·85, S.D. 0·13). This branching attern may be correlated with a respiratory function for the blood. The ain tracheae seem more permeable to the red compound of cobalt with i-nitroso-resorcinol than in insects when similar tracheal injection prepara- ons are made of both types of arthropod.

TABLE 1

Material	*Number of forks measured*	*Sum of the cross-sectional areas of the branches divided by the cross-sectional area of the main trachea*	*S.D.*
ergal tracheae of 3rd instar *Rhodnius*	25	1·00	0·16
,, ,, 4th ,, ,,	25	1·00	0·17
,, ,, 5th ,, ,,	24	0·97	0·15
bdominal tracheae of *Ephestia* larvae	31	1·02	0·18
racheae from last instar *Calliphora* larvae	31	1·00	0·13
racheae from the nymphal wing pads of *Periplaneta*	28	0·99	0·10

The structure and formation of the tracheal cuticle in *Rhodnius* have been escribed by Locke (1957, 1958). Growth and moulting of the tracheal system n *Rhodnius* have been studied by Wigglesworth (1954). He found that new racheae and tracheoles were added terminally to the tergal tracheae at each noult. If the increase in cross-section of the main trachea were equal to the otal cross-section of the new terminal tracheae, a system branching in the vay described above would result. Wigglesworth also described the great lasticity of the tracheal system, which responded by increased growth at noulting to the stimuli of low oxygen tension or the implantation of organs vith a high oxygen consumption. Thus it is unlikely that the dimensions and attern of branching are predetermined at an early stage in development. Now it is not known if this artificially-stimulated terminal growth results in system branching normally as described above, or whether the effect is a local ne only. If the branching pattern after artificially-stimulated growth is a ormal one, then terminal growth must be co-ordinated with that of the main racheа in any one branching system.

The problem, then, is to account for the maintenance of the branching attern in the system as a whole when terminal growth may vary under ifferent conditions, and to relate this to the stimulus to growth given by low xygen tensions. Experiments of two kinds have yielded information: (1) nterference with normal growth by surgical operations upon tracheae, and 2) interference with normal growth by alterations in the external oxygen ension.

Material and Methods

At 25° C 4th instar larvae of *R. prolixus* Ståhl moult 14–15 days after feedin Operations were performed upon the tracheae of newly-fed 4th instar larv and the effect observed upon the 5th instar tracheae. The distended integ ment is thin and transparent after feeding, with few muscles to hide t stretched tracheae. A fine glass hook was inserted through the integume with a quick flick to cut the tracheae, leaving a barely visible wound in t integument. The tracheae are under tension and the cut ends spring aw from the wound of entry. Preparations made 14 days after feeding show tv sets of tracheae, the old 4th instar tracheae being still in place within the ne system formed around them (see fig. 8, A, opposite p. 383) (Locke, 1958. The usual procedure was to dissect under Ringer and to fix and stain befc removing the insect from the dissecting dish for the preparation of a whc mount. Aqueous Bouin gave satisfactory fixation. In this way it was possib to compare the increase in diameter of different tracheae fairly accurately.

In some of these preparations the results may have been influenced by t failure of a cut trachea to separate completely from the wounded integumer To check this, operations were performed upon 3rd instar larvae and t 5th instar tracheae observed as before. The wound should have healed cor pletely by the 4th instar. The variations in growth after two moults were oft more striking and gave no cause to doubt the conclusions derived from earli experiments.

Some of the experiments upon tracheal growth in *Rhodnius* were repeat upon the larva of *Calpodes ethlius* Stoll (Lepidoptera, Hesperidae), whi deserves to be better known as an experimental insect. It occurs widely in t Caribbean, Central America, and the southern states of North America. T larvae are readily reared upon *Canna*, taking about 10 days to pass throu 5 instars. The tracheae and all the body organs can be seen clearly through t thin colourless integument. The tracheae are not under tension as in f *Rhodnius*, but they break fairly easily when pulled by a glass hook insert through the integument. There are more body-wall muscles than in *Rhodni* so that any operation tends to result in some wounding. To reduce the cor plications of wounding upon tracheal growth, operations were performed up 2nd instar larvae (they are then about 1 cm long by 0·12 cm in diameter) a the result observed in the 5th instar (about 8 cm long by 1 cm in diamete

Neither *Rhodnius* nor *Calpodes* proved very suitable insects for investigati the extra-tracheal growth which results when the environmental oxyg tension is lowered, but the hypertrophy is readily stimulated in the larvae *Tenebrio molitor* L. *Tenebrio* larvae were reared within a closed system various circulating gas mixtures, the composition being determined wi a gas analyser (Roughton and Scholander, 1943). Changes in the trach system were most easily detected after injecting cobalt naphthenate a developing the red compound with di-nitroso-resorcinol (Wiggleswor 1954).

Results

The role of oxygen tension

The experiments of Wigglesworth (1954), in which he observed increased tracheation in *Rhodnius* reared at reduced oxygen tensions, suggested that the oxygen tension within the system might play a part in determining tracheal growth. The hypertrophy was small compared with the normal variation in growth discussed under the next heading, even in oxygen tensions only just enough for survival (5%, 7·5%), but this did not exclude the possibility that even lower oxygen tensions inducing growth could be tolerated locally.

The lateral tracheae of a newly-fed 4th instar *Rhodnius* larva were cut upon each side of the right tergal trachea in abdominal segment three, together with the ventral trachea uniting it with the other side. The spiracle opening upon this now isolated system was occluded with soft wax. The effect of the oxygen shortage upon this section of trachea was observed at the next moult. The result varied in different preparations: in some the terminations had atrophied slightly and the main trachea showed signs of wounding, others appeared more normal, but none showed an increase in diameter greater than tracheae upon the control side of the animal.

Different combinations of spiracles were occluded in newly-fed 4th instar larvae to produce a varying degree of oxygen shortage. No effect could be detected upon the growth of the 5th instar tracheal system comparable to the normal variation discussed below. From these results it seemed unlikely that oxygen tension could act directly upon the tracheae to induce growth.

A varied oxygen tension had much more effect upon tracheal growth in *Calpodes* and *Tenebrio*. All the spiracles upon one side of some newly-moulted *Tenebrio* larvae were occluded with wax before they were allowed to feed and moult again. The tracheae upon the occluded side always showed much extra growth. In extreme cases the terminations became profusely tufted with fine tracheae and tracheoles with a corresponding increase in diameter of the main tracheae. Similar experiments were performed upon *Calpodes*. The results were not quite so obvious but occlusion always resulted in some hypertrophy.

A similar hypertrophy was observed in *Tenebrio* larvae reared in reduced oxygen tensions. Small larvae weighing 15–30 mg were kept in 10% oxygen in nitrogen at normal pressures for up to 40 days. Even after one moult extra tracheation could be seen in some larvae, and marked changes had occurred after three moults. The greatest hypertrophy occurred in the tracheae supplying the gut and muscles. The number and length of the finer tracheae increased and the main trunks were 2–3 times their normal diameter. A similar hypertrophy was obtained in larvae reared in air in large desiccators at half an atmosphere total pressure.

Tenebrio larvae were also reared in 50% oxygen in nitrogen. After three moults there had been a negligible growth of new tracheae and tracheoles. The tracheae had grown in length only and appeared very slender compared

with normal tracheae. Thus the metabolic activity concerned with determ ing tracheal growth is sensitive to oxygen at both high and low concentratio

The ease with which changes in the tracheal system may be induced *Tenebrio* and *Calpodes* may be correlated with the greater activity and mus development and higher oxygen consumption than in *Rhodnius* (a *Teneb* larva weighing about 100 mg consumes 50–200 mm^3 of oxygen per ho while a resting *Rhodnius* larva weighing about 20 mg consumes only 1 t mm^3 per hour). If the tissues are responsible for stimulating tracheal grow then the greater volume of respiring organs in *Tenebrio* and *Calpodes* might expected to result in readily-stimulated tracheal hypertrophy.

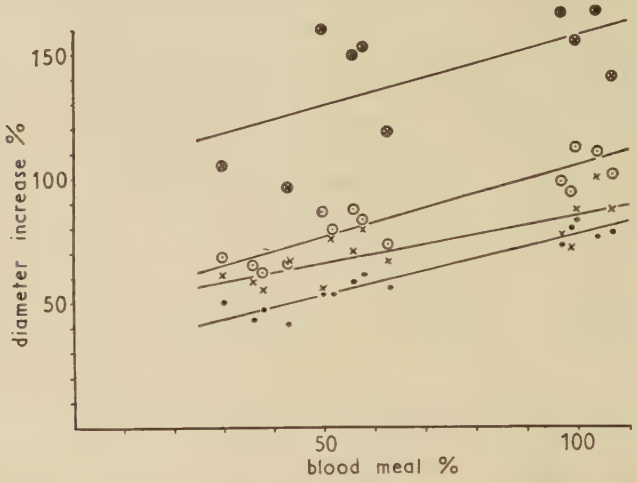


FIG. 2. The effect of feeding upon tracheal growth in *Rhodnius*. Ordinate: increase in diame at the 4th to 5th moult. Abscissa: size of the blood meal as a percentage of the maxim possible meal.

⊗ tracheae to the testis.
⊙ ,, ,, gut.
× main tergal tracheae in which the tracheae from the gut and tergites unite.
● tracheae to the tergites.

The relative stability of growth in *Rhodnius* tracheae suggested that th would be most suitable for investigating the co-ordination of growth betwe the tissues and the large tracheae.

The normal variation in growth

4th instar *Rhodnius* larvae were given blood meals of known, controll size. The larvae were weighed, allowed to feed, and reweighed immediat they were detached from the host. The size of the blood meal as a percenta of the maximum possible feed was calculated from the initial linear dim sions of the larva, and its gain in weight compared with a similar standa set of measurements for gorged larvae. The diameters of the new 5th ins tracheae were compared with the old 4th instar tracheae just before moulti The size of the blood meal as a percentage of the maximum possible feed

ised as the abscissa in fig. 2 plotted against the increase in diameter of the nain tracheae supplying various organs.

There is considerable variation in growth both with the size of the blood neal and the organ supplied. This can be correlated with the formation of new erminal tracheae and tracheoles. There is a great growth of fine tracheae and racheoles in the testis at this moult which is reflected in the increase in dia- neter of the main testis tracheae. Similarly the formation of new tracheoles n the tergal tracheae varies with the nutritional state. At the lower extreme,

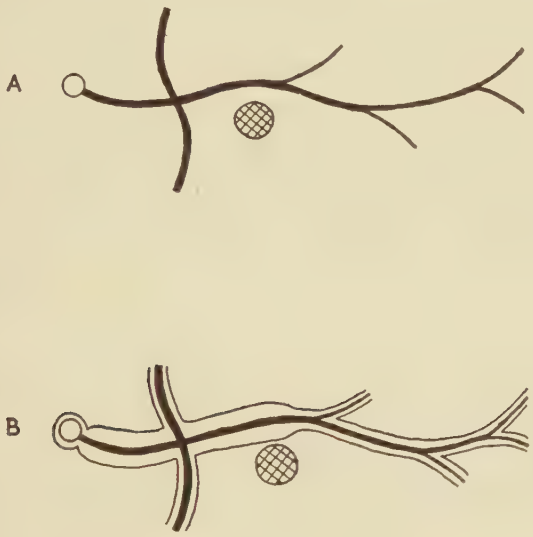


FIG. 3. Diagram to show the effect of implanting an organ with a high oxygen consumption below a tergite of a newly-fed 4th instar *Rhodnius* larva upon the increase in diameter of the 5th instar trachea. A, the tracheal supply in the 4th instar, showing the position of the implanted organ. B, the diameter of the new 5th instar trachea is indicated by a thin line round the old trachea. For clarity the new fine tracheae and tracheoles have not been represented.

vhen unfed larvae are induced to moult by grafting on to fed larvae, there is carcely any growth of new tracheae and tracheoles and the existing tracheae nly increase in diameter by about 35%. *Tenebrio* larvae moulting during tarvation behave in the same way: no new tracheoles are formed and the xisting tracheae increase in diameter by a smaller amount than normal. Presumably tracheal growth anticipates respiratory demand. Thus the pro- portions of the tracheal system are not maintained by uniform addition to the mbryonic pattern; growth in a trachea can be related both to growth in the rgan supplied and to the trachea which it joins on the way to the spiracle. For example, the tracheae to the gut increase in diameter more than the tergal racheae, while the main trachea in which they unite increases intermediately.

These observations suggest that the dimensions of the tracheal system are

determined by the growth of the ultimate branches and they in turn by t tissues.

The branching pattern after artificially-stimulated terminal growth

Normal growth in the tracheal system is co-ordinated so that the cros section of the diffusion path remains constant. It was of interest to see artificially-stimulated terminal growth also induced the appropriate grow in the remainder of the system. Growth was stimulated on the 3rd abdomin tergite of one side by inserting a 4th instar ovary, testis, or corpus allatum a newly-fed 4th instar larva after the manner of Wigglesworth (1954). At t next moult new 5th instar tracheae had formed to supply the implant. T increase in diameter of the main trachea over that upon the control side w detectable all the way back to the spiracle. Thus the proportions of the trache system as a whole are adjusted in response to new terminal growth. When t implant was inserted nearer to the spiracle only the region between it and t spiracle hypertrophied, although the terminal branches should have h a lower oxygen tension. This shows that oxygen tension has little direct effe upon the tracheae (fig. 3).

Tracheal extension and growth

The increase in diameter at moulting in many of the tracheae in *Rhodni* is proportional to the size of the blood meal. After a large meal the trache are greatly stretched and conversely they are but little extended when the me is small. From Wigglesworth's work (1937, 1940) on the multiplication epidermal cells, it seemed possible that the growth of new cuticle might va with the stretching of the trachea and the distance apart of the cells befo moulting.

Fourth instar larvae were painted with celamel cement on the edge of t abdominal tergites of one side before feeding. Other larvae were painted in t same way when fully fed and shining with distension. In this way the late tracheae were kept in a permanently distorted position while those on t other (control) side were in their natural situation. The effect of this vari extension (0–50%) was observed on the growth of the 5th instar tracheae the next moult. The increase in diameter bore no relation to the degree extension.

Tracheae were also extended in another way. The lateral tracheae betwe spiracles 2–3 and 4–5 were cut and the ends drawn through the cutic stretching the lateral trachea between them by as much as 100% over t control side (fig. 4). No extra increase in diameter compared with the cont side was detectable.

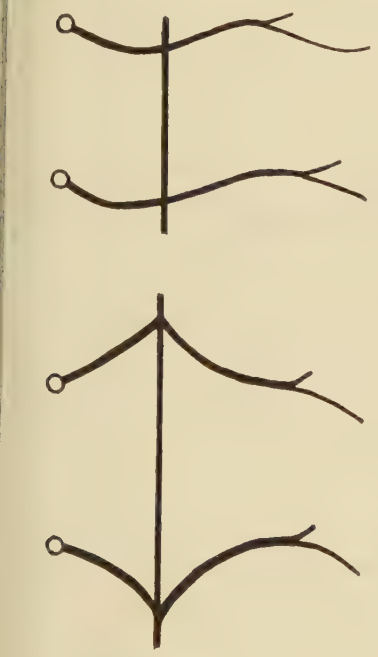
Thus, although extension may determine the future length of a trachea, t diameter is controlled in some other way.

The control of growth by the tissues

The simplest hypothesis to account for the co-ordination of tracheal growt

ch that the increase in cross-section of the main trachea is equal to the crease in cross-section of the terminal tracheae, is that tracheal growth is termined by the tissues.

To test this the tergal tracheae were cut in a segment upon one side of the domen in newly-fed 4th instar larvae. The two ends developed differently. moulting the new trachea round the end with tissue-connexions increased rmally or supernormally in diameter with a rounded end (figs. 5; 8, D, H).



G. 4. Diagram showing the arrangement of the lateral trachea between abominal spiracles 3 and 4 in a 4th instar *hodnius* larva. A, the natural position. B, e lateral trachea has been stretched by lling the cut ends through epidermal wounds in neighbouring segments.

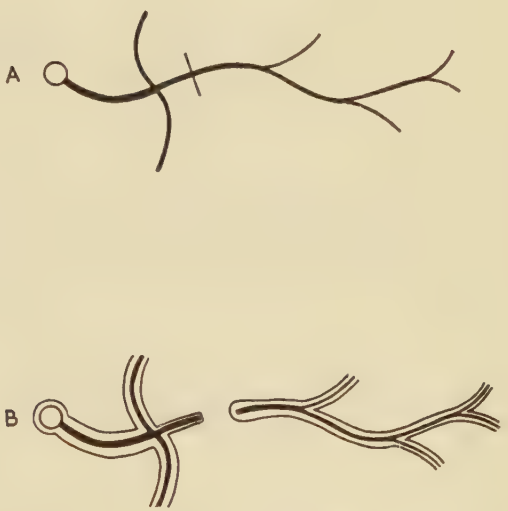


FIG. 5. Diagram to show the effect of cutting a tergal trachea in a 4th instar *Rhodnius* larva upon the growth of the new 5th instar tracheae. A, the tergal trachea in the 4th instar with the position of the cut indicated by a cross-line. B, the growth of the 5th instar trachea is represented by the new enveloping line. Compare with fig. 8, D, H.

he end connected to the spiracle showed the smallest diameter-increase mpatible with being formed round the old trachea, with a truncated end. he differences in growth cannot be attributed to wounding since they have ıd identical treatments. The two ends may have differed in the oxygen nsion available to the tracheal cells and the tissues which they supplied, ıt when a branch of the isolated tergal trachea was also cut, it too failed to crease in diameter (fig. 6). The tergal trachea was also cut between the teral trachea and the spiracle. Here the spiracle end tended to fail, while the ngth attached to the lateral trachea showed an increase in diameter, although oth should have had an adequate oxygen supply (figs. 7; 8, B).

It has been shown that hypertrophy of the terminal tracheae results in an

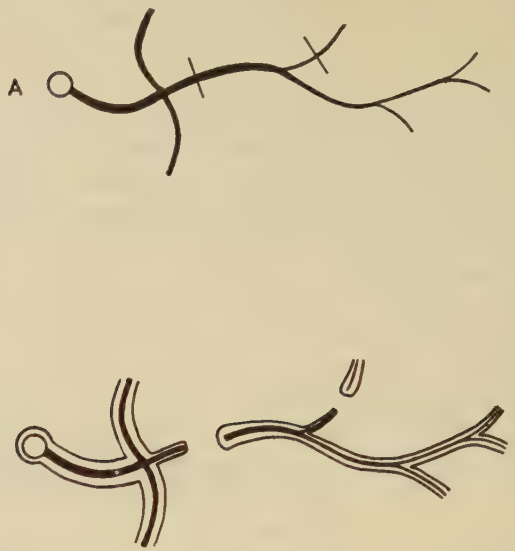


FIG. 6. Diagram summarizing the effect of cutting a tergal trachea in a 4th instar *Rhodnius* larva upon the growth of the new 5th instar tracheae. A, the tergal tracheae in the 4th instar with the position of the cuts indicated by cross-lines. B, the growth of the 5th instar trachea is represented by the new enveloping line.

FIG. 7. Diagram summarizing the effect of cutting a tergal trachea in a 4th instar *Rhodnius* larva upon the growth of the new 5th instar tracheae. A, the tergal trachea in the 4th instar with the position of the cut indicated by a cross-line. B, the growth of the 5th instar trachea is represented by the new enveloping lines.

appropriate increase in diameter all the way to the spiracle. The converse also true. The failure of a trachea cut without tissue-connexions induces partial failure in the main branch, which it joins.

Tergal tracheae were also cut in 2nd instar *Calpodes* larvae. In the 5 instar the loose end leading to the spiracle had degenerated to a small coni

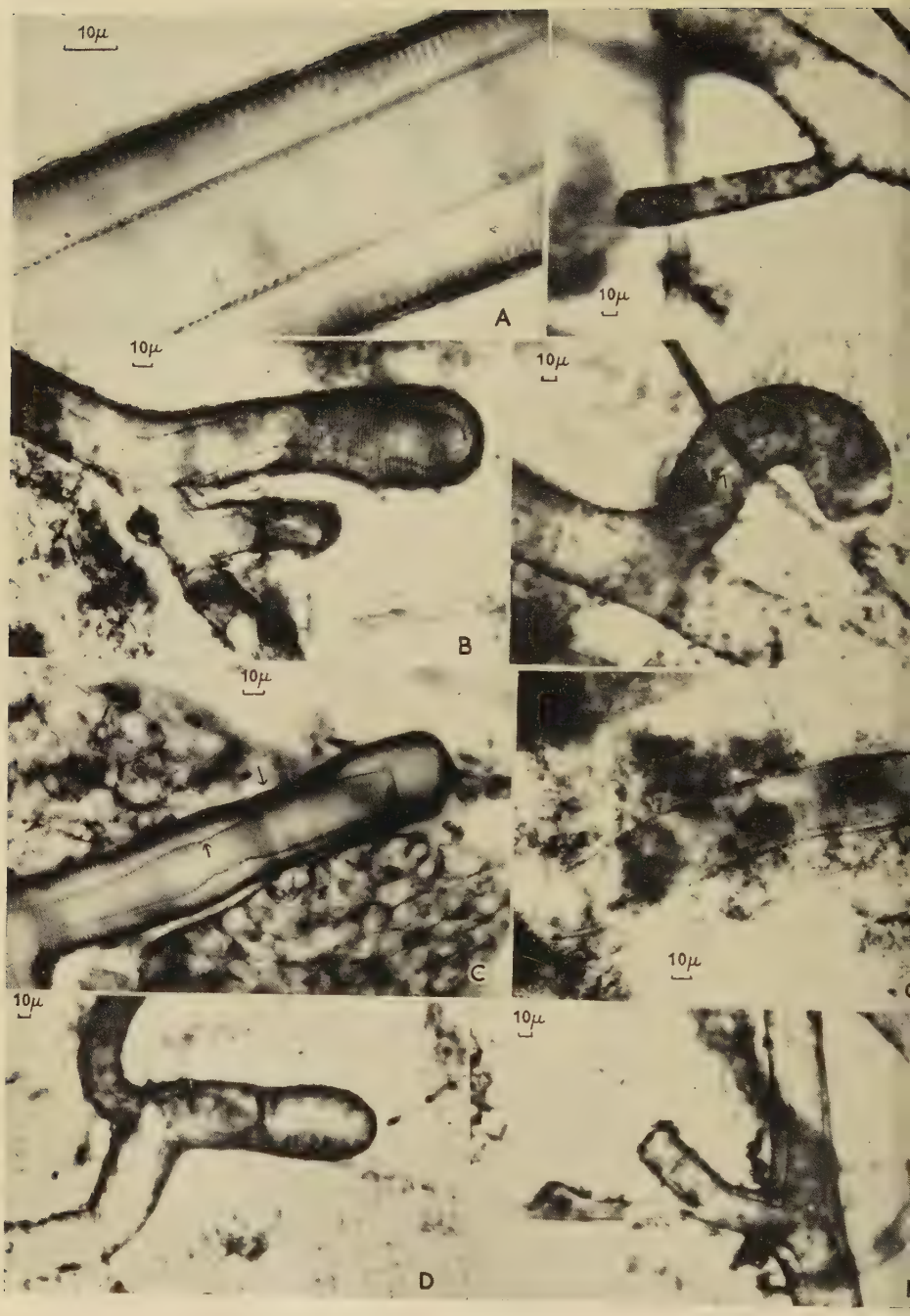


FIG. 8

M. LOCKE

ppendage. The trachea isolated in the tissues did not grow normally either. There was a resemblance to the atrophied tracheae in the abdomen of *Rhodnius* after severe oxygen-lack due to occlusion of all the abdominal spiracles. Lack of oxygen might be expected to be more severe in the muscles of the body wall in *Calpodes*.

These experiments are consistent with growth being determined from the racheal endings or the tissues.

The nodes and the control of growth on the lateral tracheae

The lateral tracheae are exceptions to the normal branching rule in that hey link up different spiracles instead of taking part in the segmental iffusion path from spiracle to tissue (fig. 1, p. 374). It is therefore of par-icular interest to study the factors controlling their growth.

At each moult the remains of the old tracheae are drawn through the spiracles and shed with the exuvium. To allow this, the lateral tracheae break at the nodes, which are predetermined weak points having a characteristic structure. The cuticle at the nodes is rucked up in an irregular fashion, interrupting the regular array of helical or annular taenidia. The effects of cutting the lateral tracheae varied with the position of the cuts relative to the node. The lateral tracheae between spiracles 2 and 3 proved most useful for these experiments because they lacked tributaries. The new trachea on the cut end containing the node increased in diameter normally or almost normally in both directions from the node. The end without the node failed to increase in diameter by the amount expected from measurements upon the control

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FIG. 8 (plate). Photomicrographs of tracheae from whole mounts of *Rhodnius* larvae. All have been fixed in aqueous Bouin and stained in Hansen's trioxyhaematein. A, B, C, D, G, and H are 4th instar larvae just before moulting to the 5th instar. E and F are from 5th instar larvae.

A, a normal lateral trachea showing the maximum increase in diameter after a large blood meal.

B, trachea from the lateral trachea to the 3rd abdominal spiracle. The trachea was cut between the spiracle and the lateral trachea, together with the lateral trachea between spiracles 2 and 3, immediately after feeding. The large trachea, which has increased in diameter, is the branch isolated from the spiracle. The smaller cut end, which has failed to grow normally, is the lateral trachea isolated without a node. Compare with figs. 7 and 9.

C, the lateral trachea between abdominal spiracles 2 and 3. The trachea was cut immediately after feeding. The trachea has increased in diameter on both sides of the node. The nodes are marked with arrows. The old trachea has been displaced slightly away from the cut end. Compare with fig. 9.

D, trachea to the 3rd abdominal tergite. The trachea was cut immediately after feeding, leaving tissue-connexions but no outlet to a spiracle. The new trachea has increased in diameter round the cut end. Compare with fig. 5.

E, the lateral trachea between abdominal spiracles 2 and 3. The trachea was cut in the 3rd instar, isolating the end shown without a node. Compare its diameter with the trachea in F.

F, the same preparation as E, showing the cut end isolated with a node. The node is close to the junction with the tergal trachea and the 3rd and 4th instar cut ends have broken there and remain in the stump, marked by arrows.

G, the same preparation as C, but showing the cut end without a node, which has failed to increase normally in diameter. Compare with fig. 9.

H, the same preparation as D but showing the cut end without tissue-connexions but with an outlet to the spiracle. The cut end has increased negligibly in diameter. Compare with fig. 5.

side (figs. 9; 8, C, G, E, F). There can be no possibility of this growth bein proportional to oxygen tension since both ends have a spiracular connexio When the cut was made through the node, damaging it, both ends tended fail. When the lateral tracheae had tributary tracheae as in segments 3–4 an 5–6, even a nodeless section increased in diameter, provided that it had a sma tributary. It was difficult to establish any quantitative relationship; even

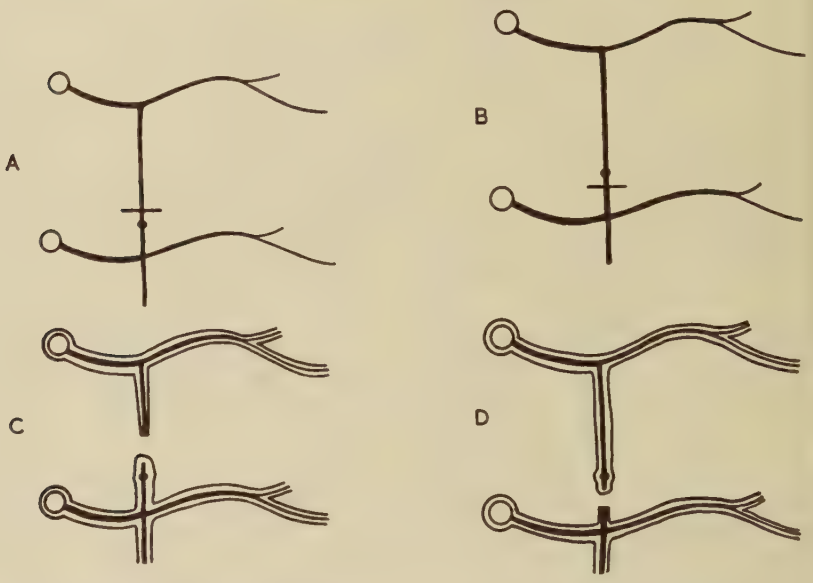

FIG. 9. Diagram showing how cutting the lateral trachea in 4th instar *Rhodnius* affects th growth of the new 5th instar trachea differently according to the position of the cut relati to the node. A, B, the lateral tracheae between abdominal spiracles 2 and 3 with the positio of the cuts marked by cross-lines. The nodes are represented by a thickening. C, D, the grow of the 5th instar tracheae is represented by the new enveloping line.

small tributary was enough to allow some growth between it and the junctio with the tergal trachea. These results suggest that the diameter of a later trachea is determined from the node in the same way that the diameter a tergal trachea is determined from the tissue-endings.

This interpretation of the part played by the node is also suggested by th marked changes in tracheal diameter which may occur on each side of it. Th lateral tracheae pass forward from the abdomen to the thorax (fig. 1). There an abrupt change in diameter at a node between abdominal segments 1 and the anterior trachea being excessively slender. This could result from diffe ential growth controlled through the node.

In the abdomen of *Rhodnius* the spiracles upon each side are connected b a ventral trachea with a node in the midline. The ventral tracheae lie betwe longitudinal segmental muscles and the epidermis, and after cutting they c not separate easily from the wound of entry. The results are therefore mo difficult to interpret and less reliable than those from the lateral tracheae. T

low for the wounding the operations were performed upon 3rd instar larvae nd the tracheae observed in the 5th instar. Cuts were made upon one side f the node. The loose ends in the 5th instar atrophied when they were ithout a node.

This experiment sometimes occurs naturally. The ventral tracheae on the rst abdominal segment often remain in two unattached halves, perhaps ecause there was a break in an earlier instar but more probably as a result of ne failure of the embryonic tracheal cells to unite in the midline. When this appens there is no trace of an irregularly buckled cuticle at the tip, but the racheae continue to grow normally at each moult. Thus the irregularly uckled cuticle is only a secondary effect of the union of tracheal cells.

The larvae of *Calpodes* were not entirely satisfactory for confirming these esults. Cut tracheae showed far more plasticity in their response, perhaps ecause of the proximity of tissues with a high respiratory demand. Many ut tracheae failed to separate from the wounded region and after 2 or 3 noults new terminal tracheae and tracheoles had arisen. When the cut section f lateral trachea was short and without a node, and when it did not redevelop ssue-connexions, its growth was very limited. In the longer ends with or ithout nodes there was often at least normal growth, but this could be attri- uted to the new tracheae and tracheoles which almost always redeveloped.

Vounding and tracheal growth

Many of the early experiments gave inconsistent results which it was elieved might be due to wounding. The effects of wounding upon the lateral racheae were observed in *Rhodnius*. The node upon the lateral trachea etween spiracles 2 and 3 usually lies very close to the more posterior spiracle, o that a cut soon after feeding isolated a long anterior end which later showed ttle increase in diameter. When this loose end was attached to the wounded pidermis by pulling the tip through the cuticle, the new trachea increased in iameter. This was not due merely to the attachment to the epidermis. When ne operation was performed on 3rd instar larvae the trachea showed little ncrease in diameter at the 4th to 5th moult although the 4th instar trachea as attached to the cuticle. The wounded region must have provided some- ning normally produced by the node. When a cut trachea complete with node as attached to the wounded epidermis, the new trachea increased in diameter ather more than normally. The wound accentuated the normal effect of the ode alone. Epidermal wounds are regions of great cellular activity with itoses and cuticle secretion which normally only occur at moulting. It is nerefore not surprising that wounding should stimulate tracheal growth. The ariation in growth of isolated cut ends may be attributable to differences in ne injury to the tracheal cells which must arise during an operation.

he polarity of the tracheal system with respect to the control of growth

It has been shown that large tracheae only increase normally in diameter hen they are connected with an organizing centre—a node, an epidermal

wound, or tracheal terminations in the tissues. This control must involv transport of the stimulus along the intervening tracheae.

When the lateral trachea between abdominal spiracles 2 and 3 is cut soo after feeding, the long anterior length lacks a node and fails to increas normally in diameter. The trachea itself is capable of growing without a noo since it does so when attached to a wound a its tip. It is not obvious why it should fai since it is attached at its other end to the terg trachea and tissue-connexions which gro normally. If the stimulus for growth in dia meter moves from the tracheal endings to th spiracles by any process similar to diffusion should be possible to alter its course to pas along the lateral trachea by severing its con nexion with the spiracle.

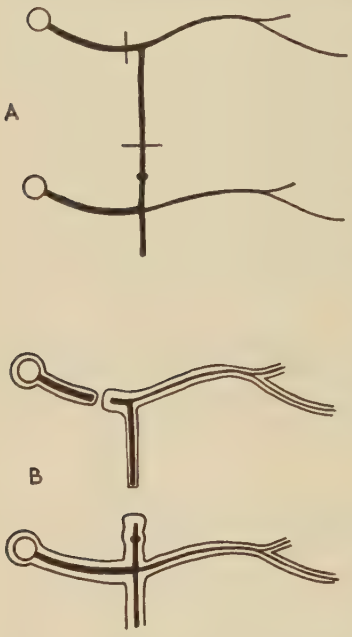

FIG. 10. Diagram of the cuts and growth of *Rhodnius* tracheae described in the text. A, the 4th instar lateral trachea between abdominal spiracles 2 and 3 with the position of the cuts indicated by cross lines. B, the growth of the new 5th instar trachea is represented by the enveloping line.

The anterior section of the lateral trache between abdominal spiracles 2 and 3 wa isolated without a node soon after feeding. A the same time the connexion with the spirac was cut as close to the junction with the terg and lateral tracheae as possible. This did no cause complete isolation from the rest of th tracheal system, for the tergal trachea connect by a slender branch to a thoracic spiracle. Th lateral trachea still failed to increase in dia meter (fig. 10).

It might have been expected that th 'strength' of the stimulus for increase in dia meter from the tergal tracheal endings coul induce a similar change to that brought abou by a small wound, but there is a difference i the point of application of the stimulus. Th leads to a concept of polarity of growth in th tracheal system. The experiment described above suggests that movemer of the stimulus causing increase in diameter can only take place in on direction. This is not due to a simple concentration gradient but is an in trinsic property of the trachea.

The time at which tracheal growth is determined

Most tracheae are almost cylindrical between branches but there may b a slight tendency to taper towards a node. This becomes more pronounce after most operations. When a cut lateral trachea is without a node and relatively short the effect is least noticeable. It is most obvious in trache attached to wounds. The trachea tapers towards the wound when no node

resent and away from it when there is a node; that is to say, the direction f taper agrees with the direction of polarity mentioned above.

Each stage of growth in the tracheal system begins at the spiracles and noves inwards towards the tissues. When the new cuticle is expanding, the ateral tracheae are about half a day behind the spiracles, and the tracheal erminations are about one day further in arrears. Presumably the time at which the future diameter is fully determined follows the same sequence. A wound stimulus to tracheal growth may also have to follow this timing.

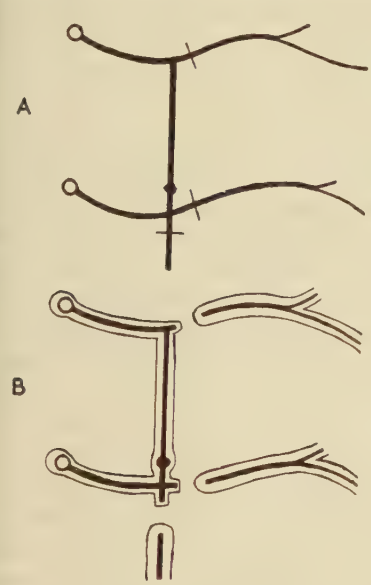

FIG. 11. Diagram of the cuts and resulting growth in *Rhodnius* tracheae described in the text. A, the 4th instar lateral trachea between abdominal spiracles 2 and 3; the positions of the cuts are marked by cross-lines. The node is marked as a swelling. B, the growth of the new 5th instar trachea is represented by the enveloping lines. Growth of the lateral trachea is unaffected by isolation from tissue-connexions.

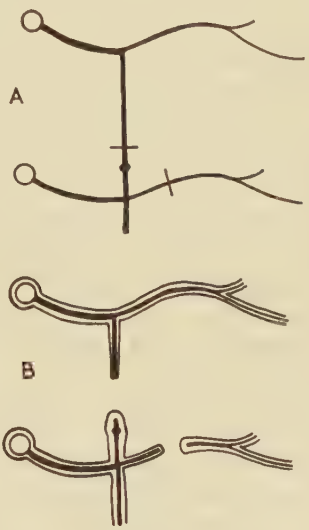

FIG. 12. Diagram of the cuts and resulting growth of *Rhodnius* tracheae described in the text. A, the 4th instar lateral trachea between abdominal spiracles 2 and 3; the positions of the cuts are marked by cross-lines and the node by a swelling. B, the growth of the new 5th instar trachea is represented by the enveloping lines.

A wound acting at the tip of a cut lateral trachea without a node might then be expected to determine the tracheal diameter farthest from the wound first, at a time when the wound is most active, and the trachea closest to the wound last, when the wound reaction has almost ceased. Similarly with the polarity reversed, only the tip next to the wound would hypertrophy.

As a result of the polarity—the property by which tracheae respond to a stimulus to growth only upon the spiracle side of the stimulus—it has been postulated that tracheal growth is controlled from the nodes and tissues. It is in accord with this hypothesis that growth should be completed first farthest from the source of control.

The control of the nodes by a blood factor

Growth of the tracheal system is related to tissue growth and the size the blood meal (fig. 2). The links between feeding and tissue growth a unknown. Perhaps certain metabolites are essential and are transported bo by the blood and from cell to cell. Now the control of growth on the later tracheae by the nodes is presumably brought about in the same way as th control of growth in the rest of the tracheal system by the tissue-endings. B the nodes are isolated, suspended in haemolymph; they can only recei information about the size of the blood meal by way of the blood or by wa of the tracheal epithelium. If the nodes are influenced by way of the trache epithelium it might be expected that the pathway would be from the termina tions in the tissues.

The node with lateral trachea between abdominal segments 2 and 3 wa isolated from tissue-connexions immediately after feeding, as in fig. 1 The new 5th instar lateral trachea showed normal growth. A lateral trache was also isolated in another way, as in fig. 12. The end of the tergal trache cut from its tissue-connexions showed a more or less complete failure but th did not influence the growth of the lateral tracheae with nodes. In earlie experiments (fig. 7), it had been noticed that growth of the lateral trache was unaffected by cutting its connexion with a spiracle. Thus the variatio in growth with feeding shown by the lateral tracheae is probably mediate through the blood.

The tissues as a stimulus for tracheole formation

When the lateral abdominal tracheae in *Rhodnius* were cut and allowed t remain free in the haemocoel, no new fine tracheae or tracheoles develope to re-establish contact with the tissues. But when the tracheae were cut an attached to epidermal wounds in the 3rd instar, terminal tracheoles quit commonly developed by the 5th instar. In the caterpillars of *Calpode* operations upon the tracheae rarely resulted in complete separation of th cut end from the tissues, and it was difficult to prevent the formation of quit extensive tissue-connexions after two or three moults. In *Tenebrio* larvae th lateral tracheae are normally devoid of side branches, but stimulation b occluding the spiracles sometimes produced numerous tracheoles, many which supplied their own tracheal epithelium. Thus under the stress oxygen-lack tracheal cells not normally destined to become tracheoles ma yet do so. This occurred most frequently in lateral tracheae close to muscle These observations suggest that contact with the tissues is necessary fo tracheal cells to differentiate into tracheoles.

Discussion

The tracheal epithelium is composed of cells of one sort only, and th cuticle which they secrete has a simple annular or helical pattern which ha been attributed to the operation of simple physical forces. The only differenti tion which a tracheal cell may undergo is in the formation of tracheoles, whic

nly occurs in certain regions and may be treated as a separate problem. A trachea may grow in diameter or in length, both of which may be measured numerically. Growth in length is mainly the result of extension in the preceding instar, but growth in diameter displays several features of interest. It may be considered as a particularly simple example of a general problem. The progress of determination in an egg is progressive in time in three dimensions. Surface cuticular patterns are a problem in two dimensions, but tracheal determination is in one dimension only. Discussion at present is necessarily speculative, but it seems that as a result of the simplicity of tracheal growth further study could lead to an answer to the problem of defining determination in less abstract terms.

The tracheae are parallel-sided tubes which branch in such a way that the total cross-sectional area of the diffusion path remains constant. Growth in the tracheal system varies with feeding and the organ supplied and occurs by the addition of a variable number of fine branches with tracheoles, the old tracheae increasing in diameter by an amount sufficient to maintain this branching pattern. Several processes can be distinguished which result in the maintenance of the branching pattern—the control of growth by the nodes and tissues, the polarity of the tracheae with respect to the operation of this control, and the timing of determination.

When epithelial continuity in the tracheal system is lost by cutting, the growth of only one of the ends is affected. The trachea on the spiracle side of the cut requires something from the tissue side in order to grow normally. It seems reasonable to equate this with the control of growth which results in the total cross-section remaining constant at different levels of branching. This implies that the region in which the diameter is being determined is under continuous control from the nodes or tissues. The mechanism of control is obscure. Lack of control does not completely prevent growth, it only reduces the diameter of the new trachea. Thus whether co-ordinated in their growth or not, tracheae must moult with the rest of the animal. The control is quantitative. The increase in diameter is precisely related to the growth of new tracheae formed later.

The polarity of this control seems to be one of the most important properties of the tracheal system. It is self-evident in the tree-like branching of tracheae, but this is not invariable; for in some tracheae, particularly the lateral tracheae of *Dixippus*, for example, tributary tracheae may make T-junctions.

Determination begins at the spiracles and moves in towards the source of control. It is tempting to suppose that it follows the completion of determination in the neighbouring cuticle. The timing of determination is unaffected by breaking the epithelial continuity, unlike the stimulus for the control of increase in diameter (fig. 13). Presumably, therefore, the time at which tracheal growth is determined is an inherent property of the trachea. This suggests that the polarity of a trachea is a result of the timing of determination. If the above hypotheses are correct, the stimulus from the tissues resulting in the control of growth is a constant one to produce parallel-sided tracheae in

a normal tracheal system. This contrasts with the stimulus to tracheal growt from epidermal wounds, which dies away with time, and conical trachea result. This suggests how bulbous tracheae and air-sacs may arise: the stimu lus from the controlling end is at first low, later it rises to a peak and die away again.

It seems very probable that tracheole-formation is directly stimulated by th tissues. This would account for the specific main pattern in a tracheal system

FIG. 13. Diagram to illustrate how the quantitative control of tracheal growth is dependent upon the continuity of the tracheal epithelium in contrast to the timing of determination, which is an intrinsic property of the trachea. The small arrows represent the quantitative control and the large arrows the progress of determination with time. A, in a normal trachea. B, in a cut trachea.

determined by the embryonic arrangement and the distribution of organs and the minor variation, the result of local tissue requirements. Albrech (1953), for example, describing the locust, says: 'In most cases, it seems a though structures requiring an especially large oxygen supply achieve thi by enlargement and modification of the nearest part of the tracheal system irrespective of the morphological relations of the primitive system. . . Thus there is superimposed on the primitive tracheal metamerism a kind o functional specialisation.'

The effects of oxygen tension have proved to be a side issue to the mai factors controlling tracheal growth. Some process related to tracheal growt must be sensitive to oxygen, but the stability of growth in *Rhodnius* unde different oxygen tensions suggests that the relation is not a very direct one Even in *Tenebrio* and *Calpodes*, oxygen tension probably plays little part i determining normal tracheal growth.

It must be realized that a first study of determination, even in a system a

ble as the tracheal system, can do little but redescribe the problem in more iliar terms. The problem will be taken a stage further in a future paper n the part played by the tracheal cells.

am very grateful to Professor Wigglesworth for supervising this work, ch I began while holding an Agricultural Research Council award at nbridge, where I profited from discussions with Dr. Beament and other nbers of the Department. I also thank Mr. G. L. Underwood and Dr. F. W. Hughes for kindly criticizing the manuscript.

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servations on the Genital System and Alimentary Canal of a Lamiid Beetle, *Nupserha bicolor*

By N. DUTT

(*From the Jute Agricultural Research Institute, Barrackpore, West Bengal*)

With one plate (fig. 3)

Summary

he genital system and alimentary canal of both sexes of adult *Nupserha bicolor* s.sp. *brunnea* have been examined. In the female, the posterior end of the rectum is losed within a membranous jacket. The membranous jacket represents the tenth ment and is much more prominent than in the male. The jacket enclosing the rectal runs a short course parallel with the vaginal passage through a common chitinous sule representing the ninth segment, in which the openings of the anus and the ital passage lie. The chitinous capsule thus looks like a 'cloacal chamber'. It ppears when the ovipositor is shot out. In the male, the posterior end of the rectum s straight to the anus. The anus is located within the membranous remnant of the h segment. In the genitalia of the female, one remarkable peculiarity is observed ally in the eighth tergite of the adult female. At this level, the eighth tergite and the sal portion of the intersegmental membrane between the eighth and ninth segments superimposed upon one another and unite medially. On one side a condyle and an que socket are formed. The condyle of each side projects into the socket of the r side to form a ball-and-socket apparatus. The mechanism disappears proximally he eighth tergite. The structure is absent in the male.

Introduction

UPSERHA BICOLOR Thoms., subspecies *postbrunnea* Dutt, was recently reported (Dutt, 1952) on *Corchorus olitorius* L. (jute). Within w years it has turned out to be a major pest of the crop. The egg-laying ale is the destructive phase. Its genitalia and alimentary canal are closely ciated and genital system is characterized by peculiarities not recorded ore. Not much information is available on the genital system of lamiid tles. In this paper an account of the structural peculiarities of the genital tem of *N. b. postbrunnea* is presented.

Material and Methods

dult beetles of both the sexes were fixed in Carnoy–Lebrun fixative for ut 10 min, dechitinized in Mukerji's mixture (1937), dehydrated, cleared edarwood oil, and embedded in paraffin wax. Sections were cut at 10 μ stained in Mallory's triple stain. A 1% aqueous solution of phosphomodic acid was used. The genital system and alimentary canal of the adult tles were also dissected out under the stereomicroscope from fresh specins and stained in borax-carmine. Specimens were treated with 10% hot OH for about 10 min for the study of the genitalia.

arterly Journal of Microscopical Science, Vol. 99, part 3, pp. 393-399, Sept. 1958.]

Female Genital System

The ovarioles are five on each side. The ovarioles of each side open into short oviduct, which bends to unite with the common oviduct. The commo oviduct is short. It opens into the bursa copulatrix. The bursa copulatrix is a elongated tube bent in the form of a U. The bursa copulatrix is flexed on th

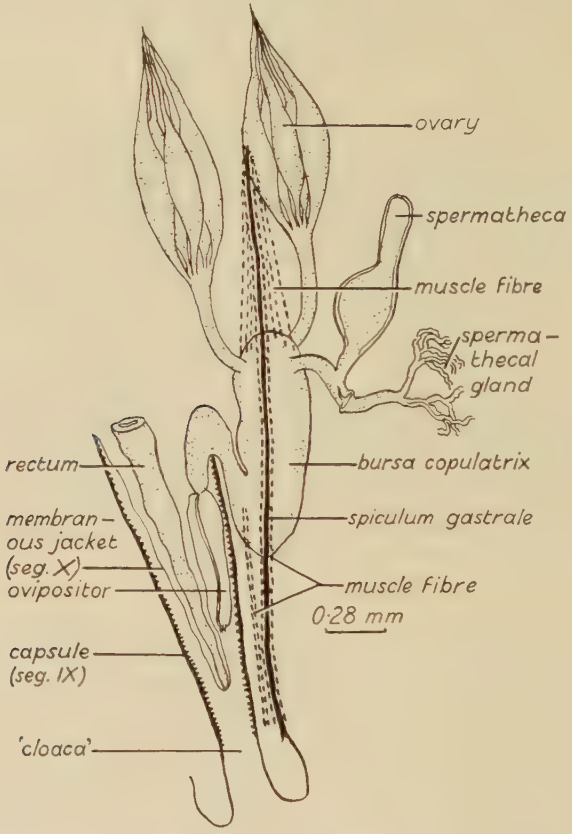

Fig. 1. The genital system of the female (diagrammatic).

ventral side, resting over the styliform rod. The terminal portion of the burs copulatrix leads into a narrow passage, the vagina. The vagina bends down t pass into the chitinous capsular sheath. The ventral wall of the capsular sheat reaches anteriorly the end of the vagina, thus separating the vaginal portio from the body of the bursa copulatrix. The capsule runs as a wide cylinder a far as the posterior end of the fifth sternite. The rectum, enclosed within th membranous jacket, passes through this capsular sheath, running parallel wit the vaginal passage. The common chitinous capsule represents the ninth ab dominal segment, in which the openings of the rectum and genital passage li (fig. 1). The chitinous capsule thus represents a kind of cloaca. The cloac

disappears as the genital passage evaginates at the time of egg-laying. Bugnion (1933) also reported a 'manchon cloacal' in the lamiid beetle *Callidium sanguineum*. The vagina lies ventrally to the rectum. The rectum reaches nearly the posterior extremity of the capsule, while the vaginal passage during rest remains at the anterior end of the capsule. The rectal jacket enclosing the

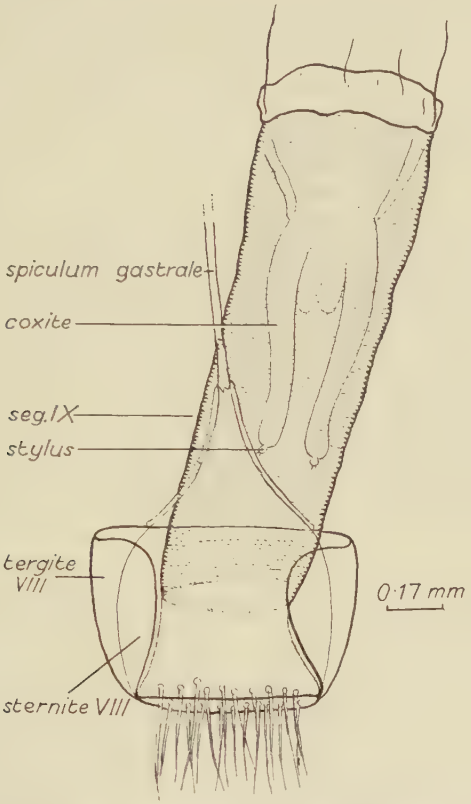

Fig. 2. The genital segments of the female (diagrammatic).

rectum is closely pressed against the wall of the vaginal passage. The appendages of the ninth segment or the chitinous capsule (coxites and styli) are borne on the vaginal end and generally occupy the middle and a little of the posterior half of the capsule at the time of rest (fig. 2). Bugnion (1933) holds the view that these appendages (ovipositor) have been derived from the tenth abdominal segment of the larva, while Tanner (1927) pointed out that only the appendages of the ninth segment persist. Styli bear sensory hairs, as in *C. sanguineum*; Bugnion (1933) named these 'antennes postérieures'. The vaginal passage, including the length of the appendages of the ninth segment, is slightly more than half the length of the rectum, which runs through the capsular sheath. The capsule narrows down at the posterior extremity to open to the exterior through the eighth segment. The eighth segment is fringed at

the posterior end with stout hairs. The eighth sternite is continued anterior like a pyramid. From the anterior end of the pyramid runs a very stou cylindrical rod with a small cavity in the interior. This styliform rod corr sponding to the spiculum gastrale (Mukerji and Bhuya, 1937) or tigel valvaire (Bugnion, 1933); it reaches as far as the third abdominal sternit Its terminal end remains free within the abdominal cavity. Longitudin muscle-fibres run along the length of the rod from the apex to the bas parallel with it. In addition there are longitudinal muscles which arise from th apex of the rod and run in an oblique direction to become attached to th common oviducal wall. A pair of muscle bands runs from the ventro-later region of the U-shaped bursa copulatrix to the eighth sternite. At the time depositing the egg, the capsule becomes evaginated and passes beyond th posterior extremity of the abdomen. At its terminal opening, the appendage of the ninth segment project to the exterior. When the capsule become everted, its internal surface becomes external as the evaginated portion slide down, leaving the rectum behind; but it carries along the vaginal portio which straightens out to form a duct leading into the opening of the everte posterior end of the everted capsule. On account of the traction, the burs copulatrix and the vaginal passage form more or less a straight line. Eversio of the capsule occurs when the abdomen contracts. Retraction occurs by th contraction of the muscle-fibres that run from the apex of the rod to the anteric wall of the common oviduct and from the bursa copulatrix to the eight sternite. The capsule is composed of a thick chitinous wall which has a serie of transverse lines dividing the chitin into narrow strips. Transverse lines a found on the internal surface of the chitin of the capsular wall; these are o the external surface when the capsule evaginates with eversion of its fold. Th lines on the capsule allow it to be crumpled in the course of eversion throug the narrow passage bounded by the eighth segment. Its dorsal and ventr walls are fused laterally and admit apically the rectal jacket, enclosing th rectum and the vagina (fig. 3, A). Posteriorly, the capsule is dorsoventral flattened and slightly depressed medially from above and below. Its later cavities look like horns. The dorsal wall of the capsule is dorsal to the rectu and lies a little ahead of the ventral capsular wall. The appendages of th eighth segment have disappeared.

One remarkable peculiarity is noted distally in the eighth tergite: there a very faint mid-dorsal line dividing the tergite into right and left halves. C examination of a cross-section this line is found to be formed by the appositio of the eighth tergite and the intersegmental membrane between the eight and the ninth segments. They lie closely superimposed upon one another a unite medially along the sutural line. On each side a condyle and an obliq socket are formed. The condyle of each side projects into the socket of t other side. The condyles fit into the sockets (fig. 3, B). This ball-and-sock

FIG. 3 (plate). A–D and F are transverse sections at various levels of the body. B is at t level of the anterior end of the eighth tergite of a female; C and D are more posterior sectio E is a longitudinal section.

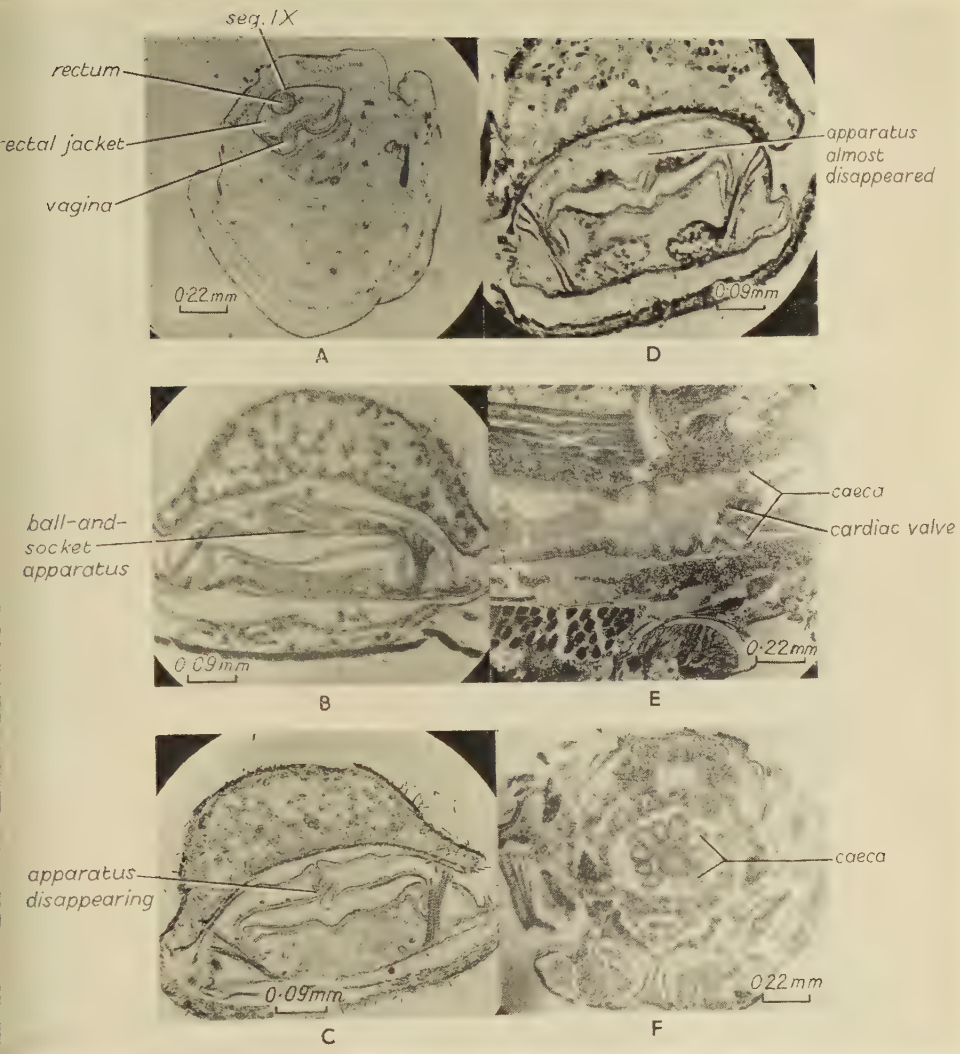
seg. IX
rectum
rectal jacket
vagina
0·22mm
A
apparatus almost disappeared
0·09mm
D
ball-and-socket apparatus
0·09mm
B
caeca
cardiac valve
0·22mm
E
apparatus disappearing
0·09mm
C
caeca
0·22mm
F

FIG. 3

N. DUTT

apparatus gradually disappears towards the posterior end of the eighth tergite (fig. 3, C, D). At this stage, the zigzag courses of the tergite and the intersegmental membrane are clearly visible. The structure is absent in the male.

Sharp and Muir (1912) have made an important study of the male genitalia of Coleoptera in general, while Tanner (1927) has made a similar important study of the female genitalia; but none of these authors has reported a mechanism such as is found in this beetle. Subsequent studies by Metcalfe (1932), Snodgrass (1935), Bissel (1937), Tanner (1943), and Bruhn (1947) have not recorded such a structure in Coleoptera. Bugnion (1933), who has made important contributions to the anatomy of *C. sanguineum* Linn. (a lamiid beetle), has not recorded such a joint. The ball-and-socket mechanism allows widening of the passage through which the capsule protrudes to the exterior at the time of evagination; it holds the capsule tight afterwards. The eversion of the capsule puts the ovipositor far beyond the tip of the abdomen for insertion of the egg into the host plant; the latter was slit by the mandibles. Dutt (1956*b*) has reported the formation of a pit by the action of the mandibles. These serve in the female as an essential accessory genital organ during the pre-ovipositional stage. The spermatheca, which has the shape of a baby's feeding-bottle, is a conspicuous structure. Its connexion with the spermathecal gland is marked by a pin-shaped chitinous valve. The spermathecal gland opens into the anterior end of the common oviduct through the spermathecal duct. Branches of the gland unite to form a common duct which is continuous with the spermathecal duct. The 'vesicule oblongue (gland colloide)', which opens independently in *C. sanguineum* (Bugnion, 1933), is absent in *N. bicolor*.

Repeated matings have been observed under laboratory conditions, though the female has a well-developed spermatheca.

The Male Genital System

The two follicles of the testis of each side are placed laterally at the anterior region of the abdomen. The first follicle lies at the level of the second abdominal sternite and the second at the level of the third. They are more or less spherical in outline, but somewhat flattened laterally. The duct, on leaving each testis, receives a duct that has been formed by the union of several ductules arising from the accessory glands. The accessory glands are elongated tubes occurring in large numbers. The duct of the testis, after receiving the duct of the accessory gland, continues backwards and the two unite to form the sperm duct or vas deferens. The vas deferens swells up to form an elongated tube, the seminal vesicle. The seminal vesicle is fusiform and is usually placed on the right side of the abdomen at the level of the third and fourth sternites. It receives a large number of fine tracheal capillaries, obviously ensuring better oxygenation of the spermatozoa stored there. The seminal vesicle continues as a narrow ejaculatory duct. The ejaculatory duct pursues a zigzag course while passing through a highly muscular bulb; it is connected with the apex of the membranous internal sac or flagellum (fig. 4). The flagellum, which is introverted, extends back as far as the junction of the bulb

and the internal sac. When the flagellum is everted, it drags out the ejaculator duct. The flagellum is chitinized and very spiny at the anterior region whe folded in. The spines are directed backwards. The horse-shoe shaped structu disappears when the flagellum is shot out by the action of three sets of musc fibres. One set connects the middle portion of the internal sac, at the level c the anterior region of the flagellum, with the struts of the median lobe. Th

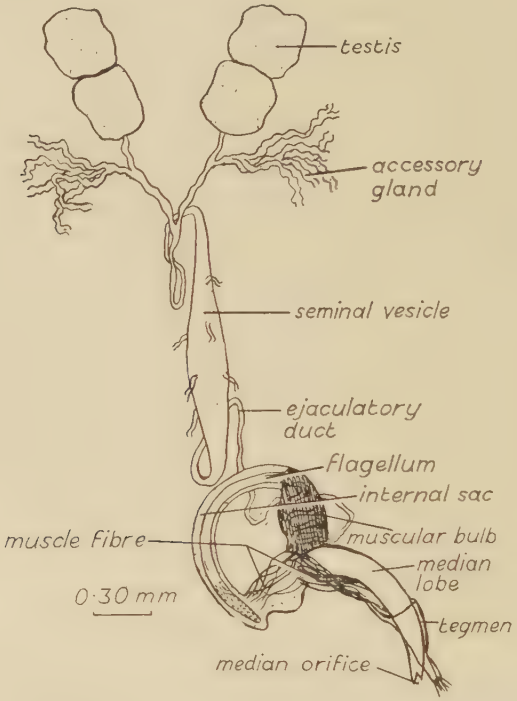

Fig. 4. The genital system of the male (diagrammatic).

other set runs ventral to the median lobe and connects it with the membranou internal sac. The third set connects the internal sac with struts of the tegmer The internal sac is evaginated through the median orifice during mating; thi has been stated by Sharp and Muir (1912) to be the general rule. The bulb i highly muscular and is connected with the internal sac that encloses th flagellum. These together bend in the form of a horse-shoe. The sac enclosin the flagellum is placed at the level of the fourth and fifth sternites. It com municates with the chitinized arched tube, or with the median lobe b a median orifice anteriorly; two struts arise from its dorsolateral edge in th posterior region. The tegmen, placed anteriorly to the median lobe, is a ring like structure with a ventral strut and a pronounced cap-piece on its dors surface. The cap-piece has two lobes united at the base. These lobes bea stout hairs posteriorly.

Alimentary Canal

The buccal cavity leads to a short oesophagus which, at the level of the suboesophageal ganglion, leads into the crop. The crop, which runs a straight course, is a voluminous sac, extending as far as the level of the second thoracic ganglion. Behind the crop is situated the simple proventriculus, which is invaginated into the anterior end of the mesenteron to form the cardiac valve (fig. 3, E). At the anterior end, the mesenteron gives out caeca (fig. 3, F). The latter are absent in the lamiid beetle *C. sanguineum* (Bugnion, 1933). These caeca are short; they are directed forwards and are applied against the digestive tube. The anterior end of the mesenteron is marked by small, wavy bulgings of its wall. The posterior end of the mesenteron bends to form a loop and then opens into the intestine. The junction between the two is marked by six long Malpighian tubules. In the mesenteron, the circular muscles are very thin and the epithelial cells are very small. The intestine is a narrow tube. It dilates in the middle and then narrows to form the rectum. The course of the rectum in the posterior end of the female is different from that of the male. In the male it runs a straight course, ending in the anus. The anus is located within the membranous remnant of the tenth segment. In the female the posterior end of the rectum is enclosed within a membranous jacket. The jacket enclosing the rectal end runs a short course, parallel with the vaginal passage, through the chitinous capsule or the ninth segment, as was mentioned before. Since the anal opening is located at the end of the membranous jacket, this seems to represent the tenth somite. This structure is much more prominent than it is in the male. The presence of such a structure has not been reported in the lamiid beetle *C. sanguineum* (Bugnion, 1933).

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A Study of the Muscular Anatomy and Swimming Behaviour of the Sea Anemone, *Stomphia coccinea*

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Summary

The sea anemone *Stomphia coccinea*, when touched by certain starfishes, frees itself from the substratum, and by a series of waving motions propels itself through the water. This locomotion has been studied by means of direct observation and by analysis of time-lapse, and normal-speed motion-picture films.

Ecological observations and stimulation tests made in the natural environment of the anemone are described and discussed.

The musculature of the anemone is described, and the function of the various muscles is discussed with regard to their participation in swimming. The muscles that play the most important part in the animal's movement through the water are the parieto-basilar and the circular muscle-sheet. The other muscles are involved in the overall reaction, but act only as aids in maintenance of the form of the anemone during actual swimming.

Experiments involving the ability of several species of starfish to elicit swimming are described. It has been established that, of those asteroids tested, only *Dermasterias imbricata* and *Hippasteria spinosa* are consistently effective in causing *Stomphia* to swim.

The possibility that swimming serves as a means of escape from starfish predators is considered. Observations and simple experiments indicate that predation may not be a factor in this relationship between starfishes and the anemone.

Observations of the anemones in their natural environment suggested that swimming might be a means to remove debris settling on the oral disk and eventually burying the animal. Experimentally, this could not be substantiated; silt is removed by the cilia of the oral end of the anemone.

That the starfish release some chemical which serves as a stimulant to the anemone is considered; but experiments indicate that if such a substance is present it probably is not water-soluble.

It was discovered that swimming could be brought about by electrical stimulation. The response is facilitated and can be controlled, to a certain degree, by giving stimuli of proper intensity and frequency for varying periods of time (e.g. 6 volts at 1-sec intervals over a period of 2 to 8 sec).

Possible uses for the swimming reaction in nature are considered, but this question still remains open to further speculation and experimentation.

Introduction

SPECIMENS of the sea anemone, *Stomphia coccinea*, dredged in the waters of the San Juan Archipelago and Puget Sound, have recently been shown to behave in a very striking and characteristic manner (Yentsch and Pierce, 1955). This anemone, when touched by certain starfishes, frees itself from the substratum, and by a series of waving motions propels itself through the water. T. A. Stephenson (1935) mentioned similar activity in *S. coccinea*, and

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recorded his observations that a specimen 'jumped' when kept under adver conditions. It is probable that the 'jumping' which Stephenson described an the 'swimming' behaviour recorded by Yentsch and Pierce are the sam Since the animals do actually move through the water, the term 'swimmin is used to refer to this activity.

The locomotion of *S. coccinea* has been studied by direct observation an by analysis of motion-picture films, both normal speed (16 frames per sec and time lapse (4, 8, and 16 frames per min). By means of gross dissection an histological sections, the arrangement of the muscles has been studied i relation to their function in swimming. Yentsch and Pierce showed that th response is elicited only by contact with a small number of species of asteroid Further studies have been made of this specificity. Entire animals, isolate parts of the body, and homogenates of various starfish tissues were teste The distribution of the anemone and the ecological conditions in whic it lives have also been studied by diving with aqua-lungs in an attempt t determine whether there is in the normal environment any possible advanta from swimming activities.

Methods and Materials

Most of the specimens used in this study were obtained by dredging i various parts of Puget Sound and the San Juan Archipelago. The type bottom in the collecting areas is sandy mud with scattered shells and rock The greater number of *Stomphia* were taken from depths of 20 to 105 fathom They were found attached to the shells of *Modiolus modiolus*, *Pododesm macrochisma*, *Pecten hindsii*, and *P. hericius*, and to rocks of various sizes an compositions. *Modiolus modiolus* is the only live animal on which *Stomph* were found. One specimen was collected on the south side of Bainbridg Island, lat. 47° 34′ N., long. 122° 30′ W., at a depth of 55 ft. It was foun attached to an empty mussel shell. *S. coccinea* is particularly abundant in Sa Juan Channel, lat. 48° 40′ N., long. 123° 03′ W., at depths of 20 to 60 fathom and in President Channel, lat. 48° 40′ N., long. 123° 01′ W., at an averag depth of 105 fathoms. Both of these areas yielded many specimens, most which were attached to *Modiolus modiolus*. Approximately 75 specimens we collected from these two locations during the summer of 1955.

Material collected during the summer and autumn of 1955 was brought t the laboratory at the Friday Harbor Laboratories of the University of Washin ton and kept in tanks supplied with running sea-water. During the rest of th year the animals were maintained in the tanks in the Oceanography Buildin University of Washington, where there is a refrigerated, recirculating se water system. Other species of animals used in the study were either dredge collected in the intertidal zone, or obtained by diving in various areas of Pug Sound and the San Juan Archipelago.

In fixing *Stomphia* for histological study, the usual difficulties of contracti and subsequent shrinkage could easily be avoided because the anemones a unresponsive to most mechanical and chemical stimuli immediately after th

wimming reaction. During this refractory period the anemones were transferred to small dishes to which $MgCl_2$ isotonic with sea-water was added. The concentration of $MgCl_2$ was gradually increased until, after approximately 30 min to 1 h, the animals were relaxed and fixatives could be added.

Several fixatives were tried, but the one that gave the best results for this study proved to be Allen's 'B' solution. A small quantity of this solution was first added to the mixture of $MgCl_2$ and sea-water containing the relaxed anemones. A few ml of the fixative were then introduced into the coelenteron by inserting the needle of a hypodermic syringe through the mouth of the anemone. After a short time the anemones were transferred to vessels containing Allen's 'B' solution and fixed for a period of 3 to 4 h.

After fixation the animals were dehydrated by passage through a series of ethanol tertiary butyl alcohol solutions. After dehydration and infiltration, the specimens were embedded in paraffin for sectioning on a rotary microtome. Sections were cut at 8 and 10 μ and serially affixed to glass slides with Mayer's albumin. The sections were either stained with Mann's methyl blue / eosin and Orange-G, or iron hematoxylin and eosin.

The motion pictures, both time-lapse and standard silent film speed of 16 frames per sec, were taken with an Eastman Kodak Ciné Special II 16-mm camera. Cine-Kodak Super-X film was used exclusively and gave excellent results. The time-lapse mechanism used is manufactured by the Electro Mechanical Development Co., Houston, Texas, U.S.A. Most of the time-lapse pictures were taken at a rate of 16 frames per min, but other speeds (noted above) were also used. A copy of the time-lapse film, *Behaviour of the sea anemone, Metridium senile*, prepared by Dr. E. J. Batham and Mr. P. M. B. Walker, kindly given to the investigator by Dr. C. F. A. Pantin, was also useful for comparative study of the action of the muscular system of the anemones. Copies of these films are available at the Department of Zoology, University of Washington.

Field Observations

At North Pass, lat. 48° 37′ N., long. 123° 01′ W. in the San Juan Archipelago, the animals occur at depths of 80 to 135 ft. Here the bottom consists of sandy mud with scattered shells and rocks. *Stomphia* lives attached to these and the anemones were usually situated so that the lower one-half to two-thirds of the column was buried in the soft bottom debris. The anemones were neither crowded nor numerous; an average of six could be seen by swimming over the area for a distance of approximately 100 m in any given direction. Other species of animals noted in the area were *Fusitriton oregonensis, Mya truncata, Schizobranchia insignis, Pseudopotamilla ocellata, Strongylocentrotus franciscanus, Modiolus modiolus, Pycnopodia helianthoides, Mediaster aequalis, Crossaster papossus, Dermasterias imbricata, Pisaster brevispinus, Ascidia paratropa, Cerianthus borealis, Metridium senile, Psolus chitinoides, Psolidium bullatum, Pecten hindsii, P. hericius, Pagurus* spp. (in *Polynices* shells), and *Aglaophenia* sp.

The only species in the above list that is able regularly to elicit the swimming reaction in *S. coccinea* is *Dermasterias imbricata*. During the study of the area a single specimen of this asteroid was found approximately 15 ft from the nearest *Stomphia*. At the time that the specimen was seen it became necessary to come to the surface and it was therefore impossible to test the capacity of this animal to stimulate any *Stomphia* in the region. In the laboratory, however, it proved to be an effective stimulator.

During later dives, other species of animals encountered at North Pass were gathered and their effect on *Stomphia* was tested. In all trials the result of stimulation was negative. The *Dermasterias* specimen previously collected during a dive at North Pass was brought along on one such occasion and was used to stimulate a *Stomphia*. The anemone released its hold on the rock to which it had been attached and swam actively for several seconds. The anemone swam, or was carried (the tides of this area cause currents up to 3 or 4 knots), a distance of about $1\frac{1}{2}$ m before it was collected and brought to the surface for transport to the laboratory.

Swimming Response: Description

The following sequence of events can be distinguished in the swimming reaction:

1. Upon stimulation the expanded animal contracts, withdrawing the tentacles and oral disk. The normal response of *Stomphia* to tactile stimulation is contraction in typical anemone fashion.

2. The anemone then slowly expands, elongates to an unusual extent, and becomes very turgid. The sphincter muscle may still be contracted enough to keep some or all of the tentacles withdrawn during this and the two following stages, or the oral disk may expand widely.

3. Frequently, but not invariably, a writhing or waving movement occurs in which the anemone whips the oral disk from side to side by flexure of the column.

4. The animal then releases its hold on the substratum and, by movements similar to those described in 3, actively propels itself through the water.

5. After swimming for periods of time lasting from only a few sec to several min, the animal settles to the bottom and comes to rest on its side. Retaining the exceptionally expanded condition, the anemone is now almost inexcitable, not responding to stimuli, either mechanical or chemical, unless of extraordinary severity. Recovery takes place within 1 or 2 min and the anemone re-establishes its hold on the substratum and regains its normal upright posture.

The series of drawings in figs. 1 and 2 show specimens of *Stomphia* in representative positions of the activities described below.

Muscular System: Description

In *Stomphia*, as in any actinian, the musculature and mesogloea together with the fluid contents constitute a hydrodynamic system (Batham and Pantin

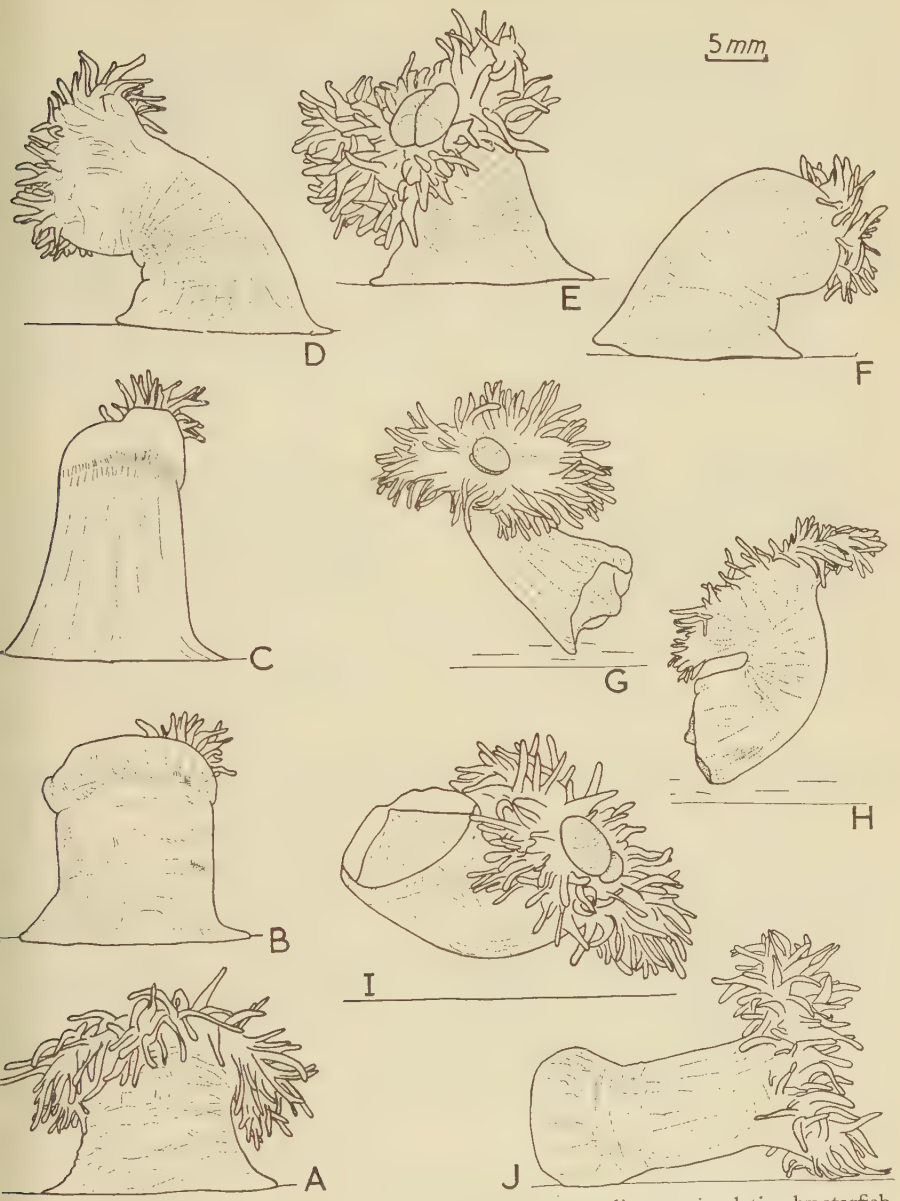


FIG. 1. A, normal appearance of *S. coccinea*. B, specimen responding to stimulation by starfish. Oral disk is partly retracted and sphincter is contracting. C, extension of column after contraction of column. Oral disk is beginning to expand. Both actions due to contraction of circular muscle sheet of column. D, lateral bending movement caused by rapid contraction of limited area of parieto-basilar muscles. E, lateral bending toward observer. Note folds of gullet protruding through mouth as result of increased internal pressure. F, lateral bending. Note raised edge of pedal disk. G, swimming. Specimen is free from substratum and is actively swimming. G, H, and I illustrate the side-to-side bending movements. H, swimming. I, swimming. Note conical shape of pedal disk. J, anemone during period of inactivity after swimming. Note that the oral end of the animal is supported by the turgid oral disk and tentacles.

FIG. 2. A, anemone beginning to right itself. Note expanded oral disk. B, righting action Approximately one-half of pedal disk re-attached to substratum. C, righting activity almos completed. D, completely re-established normal position.

1950, 1951), since the muscles work against the fluid in the coelenteron. In each of the three functional regions of the anemone—column, basal disk, and oral disk—the muscles are capable of independent activity, but under certain circumstances may work synergistically. Through the contraction of the circular muscles and the tonus of the longitudinal muscles, the anemone is maintained in columnar form. Superimposed on this activity are the co-ordinated contractions of the various muscles which are responsible for the changes in shape. The muscles are here listed.

COLUMN

Body-wall musculature

Circular muscles. In the column there are two antagonistic muscular components, circular and longitudinal, arranged at right angles to each other. A circular sheet of muscle surrounds the column, lying just medial to the mesogloea (fig. 3, A). This sheet of fibres is fundamentally continuous, but is interrupted wherever the mesogloea protrudes into the septa. Contraction of the circular muscles causes elongation of the column and, if the sphincter is contracted, an increase of turgidity.

Sphincter muscle. Derived from and included in the circular muscular system is the sphincter muscle (fig. 4, A, B). In *Stomphia* it is of the diffuse mesogloeal type, which is defined by Hyman (1940) as '. . . composed of bundles of muscle fibers or cavities lined by muscle fibers embedded in a great thickness of mesogloea'. Functionally it is very different from the rest

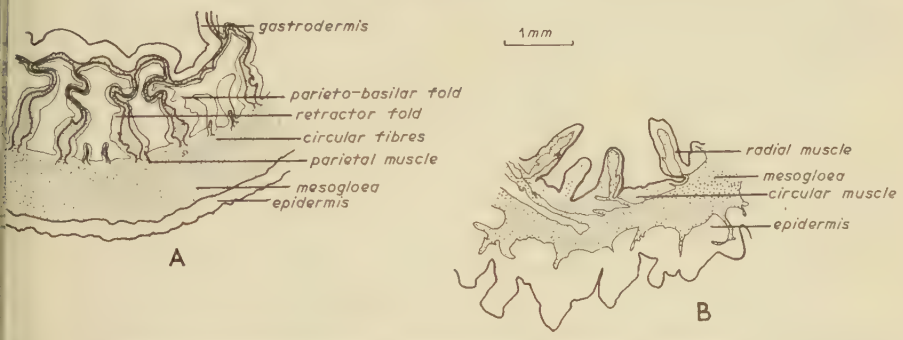


FIG. 3. A, cross-section through lower part of column. Note size of circular muscle-layer of column in comparison with that in region of the sphincter. B, cross-section of pedal disk.

of the circular system, and therefore might be considered as physiologically separate. Its contraction closes the top of the column after the oral disk and tentacles have been retracted.

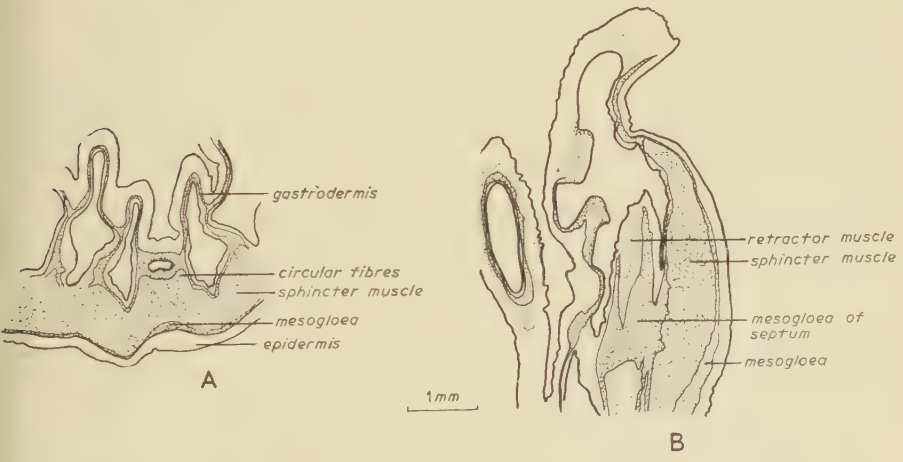


FIG. 4. A, cross-section through the sphincter muscle at the level of the oral disk. B, longitudinal section of upper column.

Septal musculature

Parietal muscles. The longitudinal muscles of the column are the parietal muscles. These are not a part of the column wall proper, but lie along both sides of all the septa near their bases. The parietal fibres lying on the endocoelic face are inserted radially into the basal disk of the anemone. Owing to the fibre arrangement (fig. 5), contraction of the parietals causes the vertical

stress in the column, resulting from contraction of circular muscles, to be distributed on to the pedal disk.

The fact that these two components of the column musculature are not superimposed upon one another, but are separated in space, conveys a definite mechanical advantage. Since the circular layer is essentially continuous and

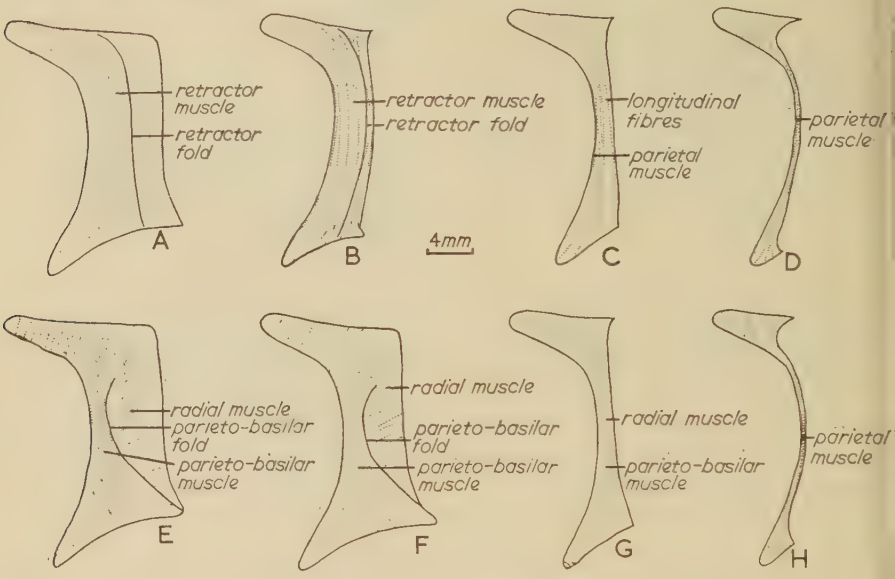


FIG. 5. A–D, endocoelic faces of septa, showing muscle-fibre pattern. A, perfect septum. B, imperfect septum bearing retractor muscle. C, imperfect septum, no retractor. D, microcneme. E–H, exocoelic faces of septa, showing muscle-fibre pattern. E, perfect septum. F, imperfect septum, with retractor (on opposite face). G, imperfect septum, no retractor. H, microcneme.

external to the longitudinal fibres which are arranged in separate bands, the probability of the two 'buckling' (Batham and Pantin, 1951) at right angles to each other, thereby raising serious mechanical difficulties, is eliminated.

Muscles of the perfect septa

Retractor muscles. Three types of septa are present in *Stomphia*, as in other actinians. The perfect septa, those that connect the body-wall to the pharynx, bear well-developed longitudinal muscle-bands, the retractor muscle, on the endocoelic face. The fibres fan out above on to the oral disk, and below on to the pedal disk, thereby connecting the oral and aboral extremities of the anemones. Upon rapid contraction the retractors pull the edge of the oral disk inward and downward. At the same time the radial muscles of the oral disk contract. After retraction of the disk, the sphincter also contracts, closing over it. The retractors aid the parietals in maintaining the shape of the column.

Radial muscles. On the exocoelic face, the perfect septa bear a relatively weak group of muscle-bands, the radials (transverse muscles of some workers), the function of which is to open the pharynx, and thus help to control the

mount of fluid in the coelenteron. These are less extensive on the directives, here the retractors are exocoelic.

Parieto-basilar muscles. The rest of the exocoelic face contains the stronger arieto-basilar muscles. These fibres originate on the pedal disk and have their nsertion on the lower two-thirds of the body-wall. This muscle is relatively nick and bears a fold at its inside edge, similar to the edge of the retractor on he opposite face (fig. 5, A). Contraction of portions of this system produces ending of the column.

Muscles of the imperfect septa

The imperfect septa that bear a retractor muscle are similar to the perfect eries in the muscles they support and in their functions. The primary morhological difference is that they do not join the body-wall to the pharynx, nd thus the radial elements do not have the function of their counterparts on he perfect septa. The radials of these imperfect septa (figs. 5, F, G) are ossibly utilized in controlling the volume and surface area of the coelenteron.

Muscles of the microcnemes

The microcnemes are small, imperfect septa bearing no retractor muscle, ut with a parietal muscle developed in proportion to the size and age of the nicrocneme. It will be remembered that the other types of septa also have arietal muscles; but in the younger septa these are of greater importance, ince other muscle elements are not yet developed. In spite of their small imensions, the many cycles of these septa supply the body-wall with its ffective longitudinal muscular system.

Pedal Disk

The muscular components of the pedal disk include a sheet of gastroermal circular fibres continuous with that of the column; radial fibres lying n each side of the attachment of the septa contributing them; parieto-basilar nuscles, and fibres derived from the retractors.

The foot is the organ of adhesion. Contact with the substratum is mainained by the parieto-basilars and the basal elements of the endocoelic longiudinal fibres, which produce a tonus in opposition to the pressure of the oelenteron. Time-lapse motion-pictures of detached animals and of animals ndergoing reattachment, show clearly the action of these muscles. In conunction with relaxation of the basilars, contraction of the endocoelic longiudinal fibres causes an inward arching of the pedal disk that will necessarily evelop suction. Maintenance of attachment is also aided by the epidermal ells of the pedal disk (Batham and Pantin, 1950, 1951), which secrete a uticle-like substance.

Release from the substratum is accomplished by contraction of the radial nuscles of the pedal disk, the basilars. The contractile state of the circular nuscles also affects the suction below the pedal disk. Other movements

associated with swimming cause the anemone to move from its place o attachment.

Oral Disk

The third region of the anemone, the tentacles and oral disk, also contain a circular sheet of muscle continuous with that of the rest of the animal, an a radial set of muscle-fibres, also medial to the mesogloea. The muscles of th disk and tentacles are important in feeding activities, but their role in swim ming is only passive. They play some part in aiding the retractors, sphincter and circular layer of the column in the retraction stage, and in maintainin the turgidity of the column.

Muscular System: Function in Swimming

When suitably stimulated, the anemone contracts and withdraws the ora disk and tentacles. This action is a very common response in actinians an results from contraction of the parietal and retractor groups of muscles. A soon as these muscles have withdrawn the disk and tentacles, the sphincte contracts, covering the entire oral end of the anemone. During this overa contraction it sometimes may be necessary to release some of the fluid con tained in the coelenteron. This is done by the radial muscles of the sept contracting to open the pharynx.

After overall retraction of the oral disk and shortening of the column, th animals elongate and often expand the disk. This is accomplished by th circular muscle sheet of the column acting against the coelenteric fluid.

In these movements, the functional integrity of the animal as well as th activity of its three morphological regions are well illustrated. The circula muscles in all parts are essentially continuous, but in this case can readily b seen to act quite independently; the two disks at opposite ends of the colum are inactive while the column elements contract strongly.

The waving motion that often occurs at this point in the reaction is th result of a co-ordinated contraction of fibres of the parieto-basilar system. T produce the lateral bending on first one side and then the other relativel small areas are involved at any one moment.

The release from the bottom is accomplished principally by contraction o the basilar muscles of the pedal disk. The circular muscles undoubtedly pla an important part in this action also. Their relaxation would reduce th suction exerted under the foot, thus facilitating the release.

In released animals the pedal disk often has a definite conical shape. Th is due to the contraction of the longitudinal fibres and the parieto-basila muscles of the septa. These fibres are probably still in the contracted stat that aided in the release of the anemone from the substratum. The swimmin itself is done by the action of the parieto-basilars in the manner discussed i the section describing the waving motion. Possibly a localized relaxation o the circular sheet at the point of bending aids in these movements. Th re-establishment of turgidity which results from the recovery of tonus in th

rcular muscles accounts for the extension of the column that occurs between ch of the bending movements. During the latter parts of the reaction the neral body tonus is maintained by the action of the circular and parietal uscles and the longitudinal elements of the septa.

When swimming ceases, the animal becomes inactive; and as stated above, is usually unresponsive to stimulation during this time. The animals will, evertheless, soon recover and, bending the column, place a portion of the ot against the substratum. Then, by localized contraction of the parieto-asilars and the basal parts of the longitudinal endocoelic fibres, the pedal isk once more slowly becomes attached to the bottom and the anemone lifts self into an upright position. The action of these muscles is preceded and ded by the relaxation of the basilar muscles of the foot.

Experimental Procedures

Since *Stomphia* was first seen to swim as the result of contact with star-shes, several species of these were tested to determine their effect upon the nemone's behaviour. In preliminary tests it was established that (for those

Table 1

Specificity of reaction

Name of starfish used as stimulus	*Number of positive responses*	*Number of negative responses*	*Number of trials*
rossaster papposus . .	1	69	70
teraster tesselatus . .	0	8	8
ippasteria spinosa . .	several hundred	0	several hundred
enricia leviuscula . .	0	53	53
eptasterias hexactis . .	0	23	23
rthasterias koehleri . .	0	48	48
olaster stimpsoni . .	2	46	48
vasterias troschellii . .	0	47	47
isaster ochraceus . .	0	25	25
isaster brevispinus . .	0	26	26
ermasterias imbricata .	several hundred	few	several hundred

arfishes that cause a positive response) any part of the entire starfish brought nto contact with any part of the anemone would usually elicit the same sponse. Therefore, in the tests in which entire starfish were used, no effort as made to limit the parts, e.g. the tube feet, epidermis, &c., used to stimulate ne anemone. Table 1 summarizes a series of tests made in an attempt o determine whether the reaction is specific for particular species. Many nore tests than actually appear in the table were made in preliminary udies; none of these gave results in disagreement with the following data. o be classified as 'positive' the anemone had to release from the substratum nd swim.

As is clear from table 1, whereas negative responses were obtained from xperiments with a majority of the starfishes tested, positive responses greatly

exceeded negative responses in the cases of two species, *Dermasterias imbrica* and *Hippasteria spinosa*.

At first, the swimming of the anemones seemed to be an avoiding reactio elicited by certain starfishes. The possibility that the behaviour serves a defence mechanism against predators was therefore considered. To te this, specimens of the anemone and various starfishes were placed togeth in a confined area. The space was limited to increase the possibility of the coming into contact with one another. The starfishes tested included *Derma sterias imbricata*, *Hippasteria spinosa*, *Crossaster papposus*, *Solaster stimpson Pisaster ochraceus*, *Henricia leviuscula*, *Evasterias troschellii*, and *Leptasteri hexactis*.

In all tests, except those involving the first two species listed above, th results were negative. In some of these tests the starfishes were seen to crav over the anemones; except in the instances involving *Dermasterias* and *Hippa steria*, this usually did not disturb the anemones enough even to caus retraction. On contact with either of these two starfishes the anemones usuall swam. Since swimming anemones have no control over the direction of move ment, they often swim in such a manner that the activity repeatedly bring them into contact with the starfish. This was not entirely due to the confine area, because the phenomenon was noticed quite often in other tests involvin much more space for movement. Also, the movements of the starfish wer completely random and they never appeared to seek out the anemones. Eve though the space available was only approximately 1½ cu. ft., the time require by a starfish of 8 to 10 in. in diameter to come into contact with a *Stomph* was often more than a week.

The possibility that swimming is used as a means of getting rid of botto debris in the event that anemones become covered by mud and silt was teste experimentally by allowing them to become attached to the inside of batter jars filled with sea-water, and then adding enough mud collected from the normal habitat to cover them partially. The mud was then stirred up b shaking the container or introducing a fast stream of water, thus partly bury ing the anemones. In no test were complete or even partial swimming move ments noted. Mud particles were removed from the upper part of the colum and from the oral area by ciliary action. By extending and contracting th column a few times, the anemones succeeded in establishing themselves i a condition not unlike that observed in their natural surroundings.

The possibility that the starfishes give off a substance that causes th anemones to swim was considered. That a water-insoluble substance on th surface of the asteroids elicits the response was suggested by the fact tha *Stomphia* swam when touched by the experimenter's fingers after a *Derma sterias* had been handled. This starfish produces a slimy substance which easily transferred to other objects that come in contact with the asteroid. series of tests (table 2) showed that positive responses could be obtained sterile gauze wrapped on the end of a clean glass rod was first rubbed o a 'leather-star' (*Dermasterias*) and then used to touch the anemones. As a cor

ol, the gauze was similarly applied without being first rubbed on a *Derma-erias*. Similar tests with other starfishes proved negative.

It may also be noted here that *Pteraster tesselatus*, which produced copious nounts of slime, as well as *Stichopus californicus*, which has a very slimy idermis, were completely ineffective in causing *Stomphia* to swim.

TABLE 2

Gauze stimulation

Stimulant	*Number of positive responses*	*Number of negative responses*	*Number of trials*
ontrol . . .	0	25	25
auze rubbed on *Derma-sterias* . . .	14	16	30

It seemed possible that some water-soluble secreted substance might cause wimming. To test this, *Dermasterias*, *Crossaster*, *Hippasteria*, and *Pisaster chraceus* were placed in clean, dry, porcelain pans and allowed to drain. The esulting liquid was collected and tested by application to *Stomphia* in two ays: by dipping a gauze-wrapped rod in the fluid and applying it to the nemone, and by using a pipette to squirt the fluid on to the oral disk of the nimal. Both techniques gave negative results in more than 100 trials.

Experiments were undertaken to determine if the substance that might ause the reaction is slowly released by the starfish into the surrounding nedium. To test this hypothesis, specimens of *Dermasterias*, *Crossaster*, and *Hippasteria* were placed in battery jars partially filled with sea-water. These ere then wrapped in several layers of cloth that served as bumpers and oated in the large sea-water tank to be kept at nearly the same temperature s that of ordinary laboratory conditions. After remaining for from 3 days to week under these conditions, the starfish were periodically removed and set side. Specimens of *Stomphia*, previously attached to 15-cm square glass lates, were then transferred into the battery jars. The anemones retracted he oral disk and contracted the column in some instances, but gave no ndication of the swimming reaction.

The water from the battery jars was also tested by the gauze and pipette nethods described above, again with negative results.

To test further the hypothesis that a chemical is the stimulating substance, ntire starfishes and isolated parts from *Dermasterias* and *Hippasteria* were omogenized in a Waring blender and applied to the anemones in the same ay as the slime from *Dermasterias*. These trials were negative, except for wo instances which can possibly be attributed to small particles from the omogenate which dropped on the anemones. The blended material was too iscous to filter; therefore it was likely that some small bits would be drawn nto the pipette, possibly supplying a necessary mechanical stimulus to give positive reaction. Results of similar tests with homogenate of *Crossaster* were lso negative.

Since starfishes were handled in order to stimulate the anemones, it seemed

possible that this treatment might cause the secretion of a material tha initiates the swimming activity. Tests were therefore made in which th anemones were brought into contact with the starfish (the starfish thus bein the passive individual). This was accomplished by using *Stomphia* attache to glass plates and placing a starfish in the same tank in such a position tha the anemones could be brought into contact with it by moving the glass plate In this manner neither of the animals involved was handled directly. Th results of these tests is shown in table 3.

TABLE 3

Anemones touching starfish

Starfish used	*Positive*	*Negative*	*Number of trials*
Dermasterias	5	6	11
Hippasteria	5	1	6
Crossaster	0	7	7
Evasterias	0	6	6

It has been previously stated that all parts of the anemones are receptive t stimulation by the proper starfish. This was further tested experimentally b stimulating different regions of anemones in contracted and in relaxed con ditions. All other experiments recorded were done with the anemones in state of normal extension, and the starfish used as a 'stimulator' was touche to the tentacles and/or the oral disk. However, a series of trials was also ru in which the stimulus was applied to the column.

Several specimens of *Stomphia* were caused to contract completely an then were stimulated with starfishes known to induce the reaction in relaxe

TABLE 4

Stimulation of relaxed and contracted anemones by Dermasterias

Condition of anemone	*Positive*	*Negative*	*Partial*	*Total*
Relaxed; column stimulated .	4	11	4	19
Contracted; column stimulated .	0	21	2	23
Semi-contracted; tentacles stimulated	12	4	5	21

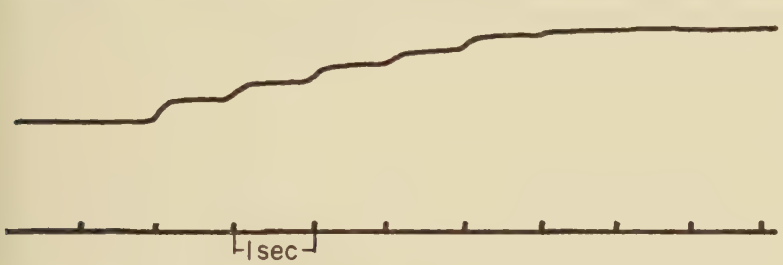
individuals. Specimens in states of partial contraction were also tested. Sta fish were brought into contact both with the column and with the few tentacle exposed (fig. 1, B). The results of one series of these tests are shown in table In some instances a specimen would begin to respond but the animal did n release; these are noted as 'partial response' wherever they occur.

The results suggest differences in the responsiveness of various regions the anemone to stimulation by *Dermasterias*. Such differences may be attribute to the relatively fewer nerve-fibres in the column as compared to those of t oral disk and tentacles (Pantin, 1935*b*); or they may be attributed to a scarci

receptors, rather than a more diffuse organization of the nerve net of the umn.

t is of some interest that release often occurred and the animal swam with- the oral disk being expanded. This was due to the fact that the sphincter l was in a state of contraction, but it was also true of some individuals that l been stimulated while in the relaxed and expanded state. In the extension ge of the reaction such animals elongated but did not expose the oral disk. is did not seem to alter the reaction beyond changing the appearance of anemone.

In experiments involving electrical stimulation of *S. coccinea*, the animals re stimulated by non-polarizable electrodes placed in contact with the base the column. This enabled repeated stimuli to be given without the animal thdrawing from contact with the electrodes. The threshold was determined



. 6. Facilitation in *S. coccinea*. Note lack of response to first stimulus, and 'staircase' resulting from succeeding stimuli. Drawn from kymograph records.

be between 2 and 3 volts. All the stimuli were given at 6 volts for 50 ms at rious frequencies, controlled by means of a Grass stimulator. The responses these stimuli were recorded on a slow-moving kymograph drum by an otonic lever attached to the oral disk of the specimens by means of a cotton read and glass hook. Thus it is primarily the actions of the longitudinal uscles of the septa that are recorded in fig. 6.

Facilitation (Pantin, 1935 *a*, *b*, *c*; Hall and Pantin, 1937; Ross and Pantin, 40; Ross, 1946 *a*, *b*, 1952, 1955; and Pantin and Passano, 1954) was ex- bited when a series of stimuli was given at 1-sec intervals. The first stimulus oduced no response, but each succeeding stimulus caused an increase in ntraction up to the point at which the animal was maximally contracted.

It was found that the various stages of the swimming reaction could be monstrated with suitable electrical stimulation. Stimulated with impulses 6-volt intensity at a frequency of 1 per sec, the following reactions were served:

2 shocks: incomplete contraction.

4 shocks: all but the tips of a few tentacles retracted.

6 shocks: complete contraction followed by extension and a few waving ovements. A long interval occurred between each of the latter, whereas in e normal reaction to starfishes they usually follow one another immediately.

8 shocks: full contraction, extension, release, with or without the waving

activity, and the animals swam actively. They also remained unresponsive fo a short time at the termination of swimming.

In order to determine if the stimuli given in the experiments describe above were the number required to produce the swimming response, th frequency was increased to 8 per sec, and a series of tests was run at 1- t 8-sec periods of stimulation. The results of this increased frequency were n different from experiments in which shocks at 1 per sec were applied to th column of *S. coccinea*; except that in one case, of the total of 6, the anemone swam after 9, instead of 8, sec of stimulation.

Analysis of Time-Lapse Motion-Pictures

The animals photographed for this part of the study were placed in a larg glass-walled aquarium supplied with running sea-water. The lighting wa constant for the 3 to 4 h required for the photographic procedure, so that th only conditions that were varied were those noted in the experiments describe below.

The time-lapse motion-pictures proved a valuable aid in studying th muscular activity of *Stomphia*. They demonstrate that the animals are i constant motion. The actions of undisturbed actinians, too slow to be other wise detectable, involve continuous movement of the body-wall and tentacle and, every now and then, a retraction of the oral disk and/or contraction of th column.

Portions of two reels of time-lapse pictures showed especially interestin activities that were provoked by appropriate stimulation. In one case, th anemone being photographed was subjected to excessive heat caused by th arrangement of the lamps used in photography. As the temperature o the water rose, the activity of the animal increased greatly. This increase i activity took the form of convulsive contractions of the circular muscula system, lateral waving due to parieto-basilar activity, and repeated retractio and extension of the oral area, for which the retractors and sphincter muscl are responsible. The edge of the pedal disk was soon raised from the sub stratum and the animal moved away from its original place of attachment. I finally re-attached itself some 60 cm distant, out of the direct rays of the lamps The time required for this movement was 3 to $3\frac{1}{2}$ h.

In another case, one of the two anemones being photographed was fed wit a bit of mussel. Feeding took place after the animals had been photographe in an undisturbed condition for about 1,500 frames at the speed of 16 frame per min. From this moment on, the activity of the circular muscle system i the fed anemone increased. This activity was very similar to peristaltic move ment, and it might be so termed. The parieto-basilar muscles and the longi tudinal muscles of the septa were also activated. The overall activity was not s intense as that elicited by excessive heat, nor were the individual movement so rapid. The unfed anemone was not affected by the activity of its neighbou It remained in the expanded condition; the only movements noted were th

onstant waving of the tentacles and a slight contraction of the column from me to time.

Discussion and Conclusions

The waving motions by which *S. coccinea* swims involve at a given moment he contraction of relatively small areas of the parieto-basilar muscle-system. ontraction of these muscles may also cause a whirling of the oral area about he oral-aboral axis of the anemone. This action appears to be the result of a ave of contraction proceeding around the column. The side-to-side motions re probably produced similarly. The difference in the two resulting activities possibly due to an interruption of the impulse as it proceeds around the olumn, or to the fact that some of the muscular elements may be in a refrac-ory state.

That the slime produced by *Dermasterias imbricata* will elicit swimming in *Stomphia* suggests that this starfish may secrete some substance into the water, r on to the anemone upon contact with it. As the experiments indicate, this ould not be demonstrated. Since none of the substances that could be ob-ained by homogenizing the material had any consistent effect on the behaviour f *Stomphia*, it can only be concluded that the reaction is not due solely to uch stimulation. Further, there was no evidence that *Hippasteria* produces substance which can be transferred to the anemone and elicit swimming.

In the experiments in which starfishes were kept in battery jars, the fact hat the anemones contracted may have been due to the necessity of their eing exposed to air for a short time during their transfer from the tank into he battery jars. None of them contracted during this transfer, and all appeared ormal upon immersion into the water which had contained the starfish. The ontraction may also have been caused by something in the water, or might ave been due to the stagnant state of the water that resulted from its not eing changed in the attempt to allow a sufficient quantity of the hypothetical ubstance to diffuse into it from the starfishes.

The experiment in which the anemones were the active and the starfish the assive participants, indicates that any substance released by the starfish that ight stimulate the anemone is not released as the result of the handling and onsequent disturbance of the animals. Considering the small number of these ests, the high number of negative results may indicate some important factor f the anemone-starfish association that is not evident at the present time. urther studies will have to be made before this question can be answered.

The experiments in this study seem to indicate that the time-interval over hich the stimuli are given is the main factor in eliciting a swimming response, lthough many more experiments involving electrical stimulation of *Stomphia* hould be done before definite conclusions are drawn. This tentative con-lusion is substantiated by the fact that stimulation by starfish usually requires period of similar duration. Positive results from electrical stimulation experi-ents also illustrate the fact that a chemical substance is not necessarily nvolved in the stimulation of swimming by *S. coccinea*.

From the time-lapse motion-pictures it can readily be seen that anemone are contantly in some state of activity. With proper stimulation, e.g. temperature increase or feeding, the activity takes the form of a definite pattern of movements that seem to be quite well co-ordinated. The best example of such co-ordination revealed by these studies is the case of temperature increase where the anemone co-ordinated its muscular activity to enable it to 'walk away' from the area of most severe heat to a spot where the environmental conditions were more suitable. It should be stressed that *Stomphia* walked away, but did not swim, as the result of this severe stimulation.

The slow activities described above for *Stomphia* (as seen in time-lapse motion-pictures) closely parallel those of *Metridium* as described by Batham and Pantin (1950 *a*, *b*). The similarity is very striking when the time-lapse film prepared by Batham and Walker and the films used in this study are compared. In the former, the responses of *M. senile* to food and the locomotion of the animal in response to certain stimuli are illustrated (Batham and Pantin 1950*a*).

Metridium is known to 'walk' without any apparent cause; but Batham and Pantin say, 'Occasionally locomotion in *Metridium* seems directly initiated in response to a prolonged adverse stimulus . . . an anemone subjected to repeated electrical or mechanical stimulation or to prolonged very powerful illumination or to the sewing-in of a recording thread would begin walking during the following night.' The anemones move over the substratum by extending one side of the pedal disk to an advanced position, attach this area of the foot to the bottom, and move the rest of the foot to the new position. The motions by which the foot is raised and advanced seem to be random but the attachment and consequent locomotion occur only in one direction. The mechanism influencing the direction in which the anemones proceeded is not understood. A complete comparative study of the locomotion of *Stomphia* and *Metridium* is lacking; but the slight differences (observed in time-lapse films) in the manner in which the two anemones move can probably be attributed to the variation in the musculature of the two species (Batham and Pantin, 1951 *a*, *b*).

The response to solid food is similar in the two species. Both anemones when given a bit of food, engulf the particle and greatly increase the activity of the circular muscle-sheet of the column (peristalsis). One apparent difference in the feeding motions of the two animals is that unfed specimens of *Stomphia* adjacent to a fed individual are unaffected by the activities of the fed animal. *Metridium*, in similar circumstances, appears to make searching movements, and peristaltic contractions are often noted in the unfed animal. A possible explanation of this variation in the activities of the two species may be the differences in the ability of the sphincter muscle in *Stomphia* more effectively to close the coelenteron and thus not allow the food juices to diffuse into the surrounding medium. Batham and Pantin illustrate an increase in the activities of *Metridium* in response to the presence of soluble food material in the water.

study of the musculature, and the observations of the behaviour of *Stomphia* l *Metridium*, indicate that actinians are capable of only a limited number different kinds of movements. But as is shown in these studies, the order which these various individual movements take place may form a definite haviour pattern which is determined by the stimulus. In the case of swim-ng, the muscles that are used and the way in which they contract are similar those in the slow, and apparently unco-ordinated, actions of the anemone rest'. But in swimming the muscular contractions are powerful, rapid, and ordinated. How that co-ordination is accomplished, and through what chanism co-ordination is achieved, remains a problem to be explored.

Throughout this investigation the most intriguing questions have concerned adaptive significance of such an activity as swimming. It is true that tain starfish are predators of anemones (Milligan, 1916); and if *Stomphia* preyed upon, its ability to swim certainly must be considered an effective ans of escape. For, even though anemones may again come into contact h the starfish during swimming, their activities help to prevent their being sped and held by the slow-moving asteroids. But all experiments designed test whether predation is a factor in this starfish–anemone relationship have en consistently negative.

Another possible use for the reaction was suggested by the fact that the emones were observed in nature to have one-half to two-thirds of the umn covered by the sandy mud of the bottom. It was therefore postulated t in the event of stormy seas or violent currents the anemones could con-vably become covered by settling debris. In such cases some method of ting uncovered, such as the writhing movements of the swimming reaction, uld be useful. In laboratory experiments the anemones uncover themselves nply by the action of the epidermal cilia; this hypothesis, then, does not count for the swimming behaviour. Therefore, for the present, the question the value of the swimming response still stands unanswered and open to ther speculation and testing.

To Dr. Dixy Lee Ray, the author wishes to express his gratitude for sug-ting the problem and for her invaluable encouragement and criticism of work. Thanks are also offered to the faculty of the Departments of Zoology l Oceanography for the use of their facilities. I am also indebted to Mrs. rraine Pilon Sund for the efficient technical assistance with the illustrations.

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Observations on the Innervation of the Integument of Amphioxus, *Branchiostoma lanceolatum*

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With two plates (figs. 1 and 2)

Summary

In this paper the pattern of innervation of one of the simplest forms of skin, the integument of amphioxus (covering the dorsal fin in the middle third of the animal) is compared and contrasted with that of the cornea and of the skin in man.

An account is given of the results of stimulation experiments in which the integument in this region was irradiated with infra-red rays.

The observations which have been made can be summarized briefly as follows:

1. No neuro-epithelial cells were encountered in the epidermis in the region chosen for study.

2. Evidence is given which suggests that the nuclei along the course of the nerve-trunks which combine to form the 'dorsal roots' of the nerve-cord are not those of the first sensory neurone. They appear to be analogous to the Schwann-cell nuclei of vertebrates.

3. The ratio of epidermal cells to parent sensory axons in randomly selected metameres of integument in the selected region is as low as 7:1. From this it has been calculated that the density of innervation is comparable with that in the cornea and in the skin of vertebrates.

4. Every epidermal cell has two or more terminal filaments ending in relationship to it.

5. Pre-terminal axons from parent axons of different diameters are widely and apparently randomly scattered beneath the epidermis.

6. Each and every epidermal cell is related to terminals which approach from different directions and serve more than one parent axon.

7. The patterned arrangement of the nerves serving the integument of amphioxus is comparable with that observed in the skin of teleost fish, in the cornea of a number of vertebrate species, and in the skin of man.

8. The nerves in the integument in the selected region can transduce non-injurious infra-red stimuli, although the stimuli were of a kind not normally encountered by the animal in its natural habitat.

9. In the light of these observations and of experimental observations in man (Weddell, 1955; Lele and Weddell, 1956; Weddell, 1957), it is difficult to subscribe to the notion that information concerning the environment is transmitted to the central nervous system from the skin by a restricted series of nerves having terminals which only transduce stimuli having quite specific physical attributes. Rather, it seems likely that information reaches the central nervous system in the form of a space-time pattern of action potentials from endings which are either more or less available to a range of stimuli having different physical characteristics. In other words, different stimuli must evoke different patterns of activity, which are analysed by the central nervous system acting in a role of an analogue as opposed to a digital computer.

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Introduction

In 1908 Parker demonstrated that four characteristic reactions can be elicited from amphioxus by the application to its integument of stimuli which in man would be expected to evoke reports of touch, warmth, cold, and pain. This led him to postulate that the receptors for mechanical, thermal, and chemical stimuli must be at least physiologically distinct from one another. In 1938, however, Ten Cate (1938 *a*, *b*) observed that, in his experience, studies confined to noting the reaction of amphioxus to stimuli which obviously link man with the changes in his environment may be a very misleading basis upon which to formulate theories as to the mechanisms underlying its behaviour.

From the neurohistological standpoint, no author has claimed that there are morphologically specialized nerve-endings in the integument of amphioxus; on the contrary, it is agreed that the nerves which do not originate in neuro-epithelial cells arise in free nerve-terminals. On the other hand, the number, size, and patterned arrangement of the sensory nerves serving the integument does not appear to have been investigated. In this paper an account is given of an attempt to fill this gap, together with the results of some stimulation experiments with infra-red rays and other stimuli which supplement the observations made by Parker and others.

Weddell and his colleagues have recently suggested that 'free' nerve-terminals in the cornea, and possibly also in human skin, are capable of transducing stimuli having different physical characteristics. Further, they submit that the patterned arrangement of non-specific nerve-terminals is the mechanism whereby environmental changes are transduced in man (Weddell, 1957). Thus, the chief purpose of this investigation was to establish that, from the histological point of view, there are no specific nerve-terminals in the integument of amphioxus and then to examine its behaviour in the light of the above hypothesis.

Literature

The integumental innervation of amphioxus has been described in terms suggesting its unimpressive nature, especially compared with that of its atrial nervous system (Holmes, 1953). It is, however, agreed that in each metamere there is a plexus of fine sub-epidermal nerves which give rise to terminals which end freely at the base of the epidermal cells. The nerves of the sub-epidermal plexus are gathered into small nerve-bundles in the cutis, which, in turn, lead into the nerve-cord through the dorsal nerve-root (Retzius, 1898; Franz, 1927).

On the functional side, Parker (1908) has shown that the movements induced by visible light only occur when the 'eye-cups' in the wall of the nerve-cord are illuminated. A narrow beam of light which illuminates the integument only is without effect. He also concluded, as the result of a

number of experiments, that the integument must contain at least physiologically distinct mechanical, thermal, and chemical receptors.

Franz (1924) confirmed Parker's (1908) observations but noted that the most effective mechanical stimulus was a natural brushing contact against the integument anywhere over the body-surface by the undulatory movements of another amphioxus in the immediate neighbourhood.

Finally, Ten Cate (1938*a*), in a series of experimental observations on animals in their natural habitat, has countered Parker's conclusions by pointing out that many of the experimental procedures employed by Parker in his studies on the animal's behaviour were too artificial to throw any light on its functional organization.

Material and Methods

Material

This was obtained from the Marine Biological Station at Plymouth during the months of September and October 1954, February 1955, and December 1956. The specimens were adults of different sizes and at different stages of sexual development. No larvae were examined. Immediately upon arrival the animals were placed in a large tank containing continuously aerated sea-water in a semi-darkened, cool room at a nearly constant temperature (17° C ± 1° C). The bottom of the tank was covered with a layer of sea-sand to a depth of 3 cm and sea-water surmounted the sand to a depth of 15 cm. Experimental observations were carried out within a week of the arrival of each batch of animals, although surplus animals survived for 3 months without any apparent alteration in size and behaviour.

Histological methods

Twelve animals were immersed for 20 min in an 0·02% solution of methylene blue in sea-water containing 50 units of hyaluronidase per ml. They were then transferred to fresh sea-water and examined under a binocular microscope.

Fifteen animals were anaesthetized with urethane immersed in sea-water containing 50 units of hyaluronidase per ml and then fixed in 10% formalin made neutral to litmus paper with calcium carbonate. After fixation they were impregnated with silver by a modified Bielschowsky-Gros technique and sections of integument were cut in various planes.

Ten animals were anaesthetized with urethane, left for 20 min in sea-water containing 50 units of hyaluronidase per ml, fixed in Bouin's solution or Susa, embedded in wax, and cut in serial section, some in the longitudinal, others in the transverse plane. The sections were stained with haematoxylin and eosin.

Ten animals were anaesthetized with urethane and tangential slices of body wall removed with a safety razor blade and placed on glass slides either in a drop of plain sea-water or sea-water containing 0·05% methylene blue.

Experimental methods with intact animals

Controls. These were carried out on 5 specimens in a quiet room illuminated by a darkroom safe-light fitted with a red filter. Each animal in turn was placed in a dish 16 cm in diameter, containing enough fresh sea-water to cover it completely when it lay still. As soon as it had come to rest, the time was noted and the animal was observed continuously for 30 min, during which time it received no extraneous stimuli of which we were aware. Any movements seen (other than the rhythmic ones of respiration) were recorded. A note was made of the time of onset of the movement, its duration, and whether or not it resulted in a change in the position of the animal. The animal was returned to the tank as soon as the experiment was over.

Stimulation. Each animal in turn was again placed in the dish and after it had come to rest, a total of not less than 10 stimuli of each of those listed below was delivered in random order to the integument covering the dorso-lateral surface of the middle third of the body; the time of onset, duration and nature of any movement evoked being recorded.

(i) A 1-inch bristle of no. 1 nylon surgical suture (Lele, Sinclair, and Weddell, 1954).
(ii) A bevelled-edged disk of hardwood having a circular area of 19·6 sq. mm.
(iii) A fine sharp-pointed needle.
(iv) A circular beam of white light, 1 cm in diameter, containing less than 5% of red and infra-red rays.
(v) A circular beam of infra-red rays 1 cm in diameter, wavelength 1 to 3 μ, energy value 0·1 to 2 cal/cm^2/sec (Lele and Weddell, 1956).

Experimental methods with operated animals

Controls. Integument covering the middle third of the dorsal aspect of each of another series of 6 animals was irradiated with infra-red rays (1·8 cal/cm^2/sec), first on one side of the mid-line, then on the other. The time between the onset of stimulation and any movement evoked was recorded.

Operation. The animals in turn were laid on cotton-gauze soaked in sea-water and the integument covering the dorso-lateral aspect of the middle and caudal thirds on one side was removed. To do this two incisions were made, one extending from the junction of the middle and cranial thirds of the animal down the whole extent of the mid-dorsal line and the other from the same level down the mid-ventral line. The anterior ends of the incisions were united and the integument lying between the longitudinal incisions peeled off. This caused the animal to wriggle around in the dish at intervals for 10 to 15 min.

Stimulation. Thirty minutes after operation and again 2 h later, each animal was stimulated with infra-red rays as in the control experiments. The lapse of time between the onset of stimulation and a movement causing displacement was recorded.

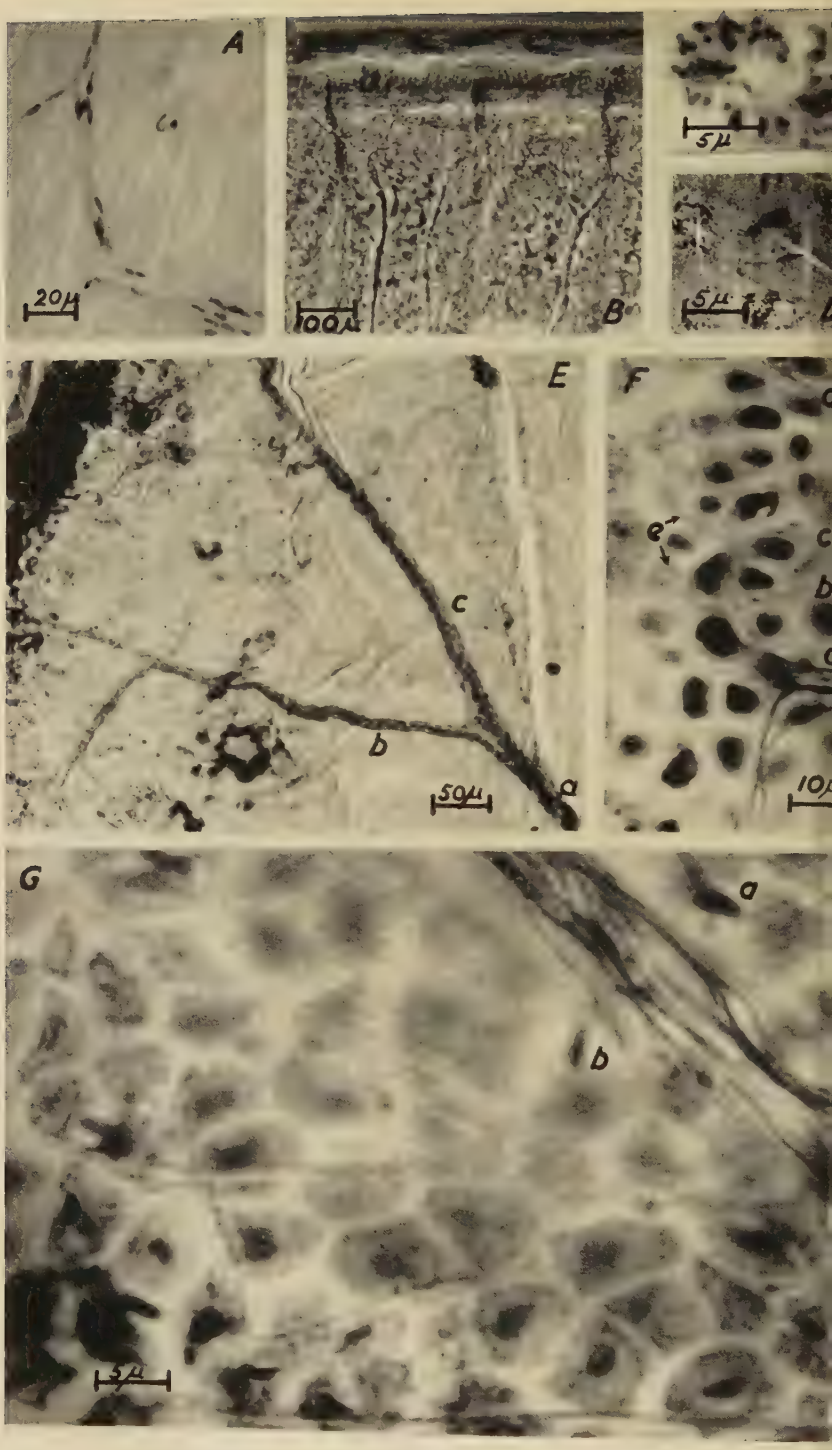

FIG. 1

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At the end of the experiment, each animal was fixed in Bouin's fluid and subsequently cut into serial transverse sections, which were stained with haematoxylin and eosin.

Histological Observations

No cells giving rise to nerve-fibres passing along bundles towards the dorsal nerve-cord were seen in the epidermis of the dorsal fin. Indeed, no cells having distinctive morphological or staining features were seen in this region in sections from active uninjured animals which had been carefully handled during their preparation for microscopical examination.

In the sections stained with haematoxylin and eosin both dorsal *and ventral* nerve-roots are seen as nucleated tracts comparable to those in vertebrates, but the nuclei are rounder and less evenly spaced than the Schwann-cell nuclei of vertebrates (fig. 1, A). The ratio of the length to the breadth of the nuclei under consideration is $2 \pm 0{\cdot}25$, whereas that of Schwann-cell nuclei in the rabbit ear is $3 \pm 0{\cdot}5$. In silver preparations, the nuclei were never deeply impregnated and no processes appeared to extend from their neighbourhood in either direction along the nerve-trunk. However, both impregnated axons and nuclei lay within a common, faintly outlined, and apparently structureless sheath.

Further to test the hypothesis that these nuclei are those of the cells of the first sensory neurone, counts were made (in relation to three different dorsal nerve-roots) of the numbers lying between the origin of the ramus dorsalis from the dorsal nerve-root and the termination in the integument of the nerves serving it. The counts were compared with the number of impregnated sensory axons entering the dorsal nerve-roots from the ramus dorsalis of

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Fig. 1 (plate). A, nucleated strands of cytoplasm related to the pathway taken by the nerve-fibres passing from the integument to the nerve-cord. Haematoxylin and eosin preparation.

B, nerve-trunks containing axons serving integument covering the dorsal fin. Photomicrograph from living animal kept in 0·02% methylene blue in sea-water for 16 h.

C, a transverse section through the latero-dorsal cutaneous nerve from the middle third of the animal. Modified Bielschowsky-Gros preparation. Note the variation in the diameter of the axons.

D, oblique section through the integument in the plane of the basement membrane. Two apertures are seen through which axons pass to the epidermis; an axon is passing through the larger opening. Modified Bielschowsky-Gros preparation.

E, methylene blue preparation showing the sensory ramus dorsalis of the mixed dorsal nerve emerging from the myoseptum (*a*). It is giving rise to a latero-dorsal (*b*) and supero-dorsal sensory branch (*c*).

F, oblique section through the integument. Collaterals from dorsal root axons can be seen in the subcutis passing towards the epidermis. There is one bundle of axons of different diameters (*a*) and three single axons (*b*, *c*, *d*). Fine axoplasmic filaments can be seen lying in between the basal aspects of epidermal cells (*e*). Modified Bielschowsky-Gros preparation.

G, oblique section through the integument; a bundle of axons piercing the lattice work of connective tissue-fibres forming the basement membrane can be seen at *a* and *b*. The sub-epidermal plexus of axoplasmic filaments is well seen towards the lower left portion of the picture. The large bundle of axons in the top right-hand corner of the picture is entering the subcutis as it passes from left to right. Modified Bielschowsky-Gros preparation.

corresponding metameres in a different animal of about the same length (fig. 1, C). The counts are given in table 1. The size of the nuclei varies little from region to region; they were $3{\cdot}0 \pm 0{\cdot}5\ \mu$ in length and $1{\cdot}5 \pm 0{\cdot}5\ \mu$ in breadth and they contained no visible nucleoli, although these were always seen in the nuclei of cells in the nerve-cord; moreover, the nuclei in the nerve-cord are larger than those in the nerve-roots. In a random sample of 20 cells the size ranged from 5 to 9 μ in length and from 3 to 8 μ in breadth.

TABLE 1

Specimens nos. 1 and 12, 6 cm and 6·1 cm long respectively

Roots	*No. of axons*	*No. of nuclei*
A	70	108
B	63	102
C	60	102

Table 1 shows the number of axons in sensory ramus dorsalis serving dorsal nerve-roots in 3 closely adjacent metameres in the middle third of specimen no. 1 and the number of nuclei in sensory nerve-trunks serving dorsal nerve-roots in 3 adjacent metameres in the middle third of no. 12.

Taken at their face value, then, these figures suggest that the nuclei are not those of the first sensory neurones.

The general features of the layout of the nervous system was studied in the whole specimens in which the nerves were stained with the methylene blue which had been added to the sea-water in which they were living. Fig. 1, B is a low-power photomicrograph showing bundles of nerve-fibres passing towards the integument covering the dorsal fin in the middle third of such an animal. It was noted in specimens stained in this way that the size of the nerve-bundles seemed to bear a fairly close relationship to the areas of the segmental integument which they subserved, being distinctly smaller in metameres in the posterior third of the animal. This suggested that some measure of the density of innervation of the integument covering the dorsum of amphioxus might be obtained by counting the number of axons contained in the supero-dorsal cutaneous nerve (one of the parent divisions of the sensory ramus dorsalis of the mixed dorsal nerve-root, see fig. 1, E) and comparing this figure with the number of epidermal cells covering one-half of the dorsal fin in a single metamere, the territory of integument which is seen to be supplied exclusively by this nerve.

In only one animal and only in relation to three different bundles was the plane of section approximately transverse and silver impregnation deep and selective enough for counts to be reliable. The numbers recorded are likely to be somewhat on the low side, for only axons about whose identity there was no doubt were included in the count. There is, however, no reason to suppose that many axons normally impregnated by silver escaped enumeration.

Further details of the innervation of the integument will be described in

elation to photomicrographs obtained from different specimens which had been prepared for examination in different ways.

TABLE 2

Specimen no. 14, length 6 cm

Metamere	*No. of axons*	*No. of epidermal cells*	*Surface area of integument (sq. mm.)*
A	20	140	0·679
B	18	137	0·636
C	16	121	0·586

Table 2 shows relationship between the number and surface area occupied by epidermal cells covering one-half of the integument of the dorsal fin of a single metamere and the number of parent dorsal root sensory axons which serve the area.

Axons passing towards the integument in the ramus dorsalis of the dorsal nerve-root vary in thickness; most of them are too fine to measure accurately so that a histogram could not be constructed. The majority of axons appear to be between 1 and 2 μ in diameter; the largest reach 4 μ and the smallest are less than 1 μ in diameter (fig. 1, C). The ramus dorsalis leaves the myoseptum at a point just above the V-bend (fig. 1, E). It soon divides without collateralization of axons into two: a small branch (approximately one-quarter of the axons), the latero-dorsal cutaneous nerve; and a larger branch (approximately three-quarters of the axons), the supero-dorsal cutaneous nerve.

Each dorsal root parent axon on entering the subcutis (which consists of a network of collagen fibres permeated by blood-vessels) gives rise to numerous collaterals which pass obliquely towards the epidermis. They proceed singly, in bundles of three or four, rarely in bundles of up to eight axons. The juxtaposition of axons of different diameters is apparently random, for some bundles contain relatively few thick axons, others about equal numbers of axons of all diameters, whilst solitary axons in the subcutis are sometimes thick and sometimes thin (fig. 1, F).

The collaterals then traverse the cutis; this consists of a fibrillary layer of connective tissue containing no nuclei situated immediately below the basement membrane and difficult to separate from it; the basement membrane is also non-cellular and made up of a lattice work of closely knit fibres running at right angles to one another. The axons pass vertically through the basement membrane (figs. 1, G; 2, A, B). The 'apertures' in the basement membrane through which they pass are conspicuous in fresh specimens of integument under phase-contrast microscopy and in selected silver preparations (fig. 1, D). The axons, still of widely different diameters, in a random mixture, emerge from the basement membrane void of visible sheaths and come to lie immediately subjacent to the epidermal cells. Here they again collateralize extensively. The branches change direction through a right angle on leaving the parent axons and travel for various distances, interweaving with and crossing one another to form a complicated sub-epidermal plexus (fig. 1, G).

The collaterals from a single parent axon vary in number and size. They diverge from one another at their origin and so become disseminated in different directions. The particular direction depends partly upon the number of collaterals which arise, for when numerous they radiate from their point of origin in a stellate fashion (fig. 2 D). Some of the collaterals (most commonly the thicker ones) give off branches while proceeding for a comparatively long distance (in one section equivalent to the sum of the breadth of 15 epidermal cells) beneath the epidermis before terminating (fig. 2, C). Others proceed for shorter distances and still others apparently terminate immediately after piercing the basement membrane. In this way, not only do terminals derived from different parent axons end in close proximity to one another but parent axons serve numerous terminals which are widely and apparently randomly scattered throughout the epidermis.

In agreement with Retzius, we found it difficult to determine, in either fixed methylene blue or silver impregnated sections, exactly how the finest collaterals terminate. However, in sections which showed the least evidence of shrinkage and/or irregularity, fine filaments appeared to end by attenuation near the plane of junction between the epidermal cells.

By phase-contrast microscopy, terminal axoplasmic filaments cannot be seen in freshly cut sections immediately after mounting either in sea-water or methylene blue in sea-water. After 10 to 20 min, parent axons, some of which are surmounted by fine thread-like extensions, can be seen (fig. 2, E). At the same time, small gaps become visible between the epidermal cells into which these filaments appear to pass, often seeming to proceed as far as the cuticle covering the surface of the integument. After 30 to 60 min, particularly in the case of specimens which have been mounted in sea-water containing methylene blue, the terminal filaments form a series of interconnected beads lying in gaps between epidermal cells (fig. 2, F). Such appearances are also

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FIG. 2 (plate). A, oblique section through the integument. Axons can be seen in the sub-cutis on the right of the picture. Some are passing through the apertures in the basement membrane, *a*, *b*. Photograph taken by phase-contrast microscopy 10 min after mounting the specimen from a living animal. Compare with fig. 1, G.

B, vertical section through the integument. Shows an axon (*a*) passing through the sub-cutis towards the basement membrane which it pierces at *b* and gives rise to sub-epidermal filaments, one of which is seen at *c*. Modified Bielschowsky-Gros preparation.

C, oblique section through the integument. Shows the long sub-epidermal course pursued by the thicker of the two collateral axoplasmic filaments. *a*, aperture in basement membrane; *b*, thick collateral; *c*, finer collateral. Modified Bielschowsky-Gros preparation.

D, tangential section through the integument. Shows parent axons giving rise to axoplasmic filaments in the sub-epidermal zone. They diverge from one another and run in a plane at right angles to that taken by their parent axons. Modified Bielschowsky-Gros preparation.

E, oblique section through the integument. Shows threadlike extensions from a parent axon as they form the sub-epidermal plexus. Specimen from a living animal examined by phase-contrast microscopy; picture taken 20 min after mounting.

F, oblique section through the integument. A swollen parent axon can be seen giving rise to a beaded filament lying in a space between two epidermal cells. Picture taken by phase-contrast microscopy 20 min after mounting the specimen (which was taken from a living animal in 0·02% methylene blue in sea-water.

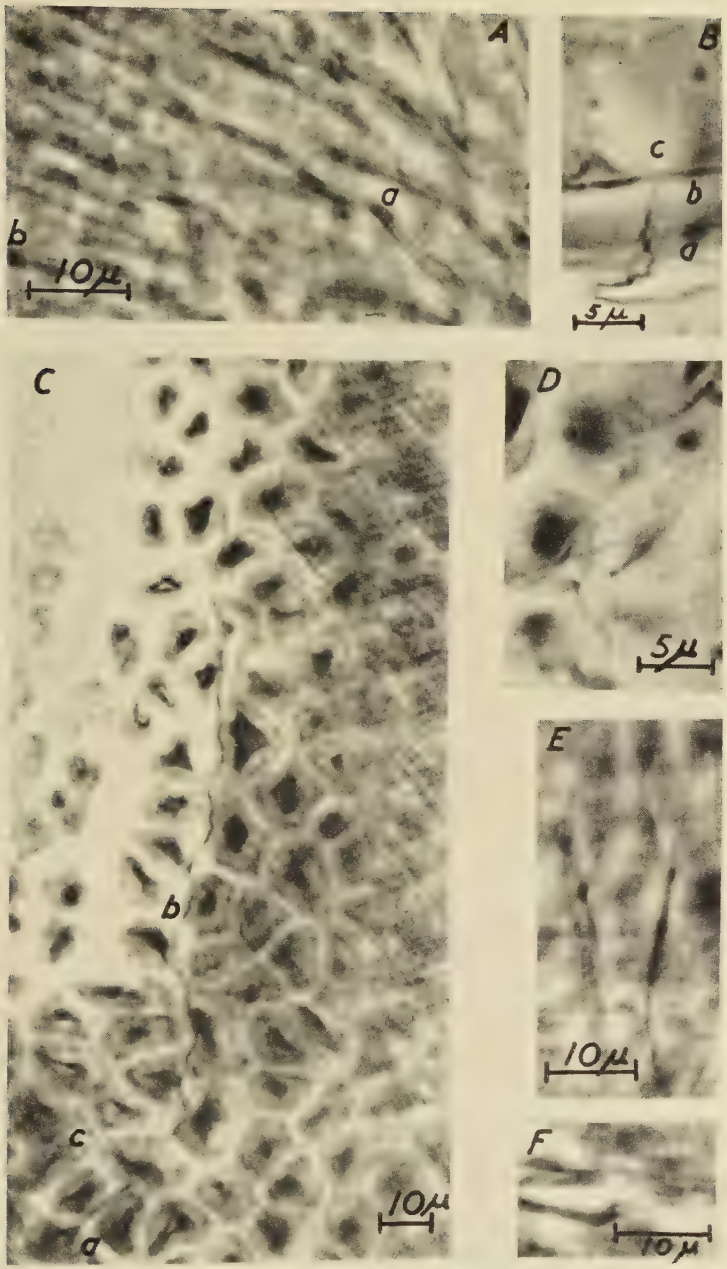

FIG. 2

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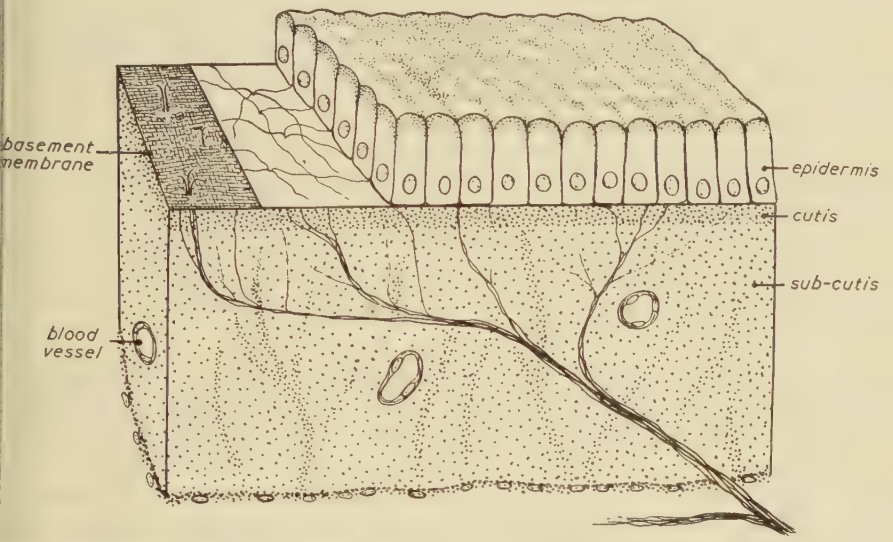
een in 'fixed' methylene blue preparations when high concentrations of dye re used.

Details of our observations on the innervation of the integument covering he dorsal fin in the middle third of amphioxus are summarized in fig. 3.



IG. 3. This summarizes in diagrammatic form the patterned arrangement of the nerves serving the integument covering the dorsal fin in the middle third of amphioxus.

Briefly, it seeks to illustrate that in relation to the integument covering the lorsal fin in a given metamere, every epidermal cell has two or more terminal ilaments ending in relation to it; moveover, pre-terminal fibres from parent xons of different diameters are widely and apparently randomly scattered eneath the epidermis. Thus each and every epidermal cell is in contact vith terminals approaching from different directions and serving more than ne parent dorsal root axon.

Experimental Observations

Unoperated animals

Control experiments. Within less than a minute of being placed in the dish, ach animal came to rest on its side. For the next 30 min the position of the nimals in the dish did not change appreciably, although some of them exhibited few feeble wriggling movements at random intervals after observation had egun. These movements, which lasted for less than 5 sec, apparently arose pontaneously but they were not vigorous enough to displace the animal. The maximum number of such movements was 4 in the 30 min during which ny of the animals was under observation. Although every effort was made o secure silence, the observer inadvertently introduced noises from time to

time but they did not appear to be connected with the movements observed. The deliberate introduction of loud noises evoked no movements.

In view of this it was decided that any movement deemed to have been evoked by stimulation of the integument would have to satisfy two criteria: they would have to be vigorous enough to displace the animal, and they would have to follow stimulation so that the correlation was significant ($P < 0{\cdot}01$).

Mechanical stimulation. A no. 1 nylon suture brought briskly into momentary contact with the dorsal fin in the middle third of the animal evoked no movements.

Light contact of the hardwood probe against the middle third of the dorsal fin and the adjacent dorso-lateral integument on one side invariably caused the animal to wriggle away from the stimulus and immediately to come to rest in a new position in the dish. A rapid brush-like motion of the probe against the integument invariably caused the animal not only to wriggle away from the stimulus but to continue to wriggle around in the dish for up to 10 sec before taking up a new position.

The brisk insertion of a fine sharp needle into the integument covering the lateral aspect of the middle third of the dorsal fin invariably caused the animal to wriggle away from the stimulus and to continue to exhibit wriggling movements for between 5 and 10 sec after the stimulus had been withdrawn. To evoke a movement the point of the needle had to pierce the integument. A blunt needle evoked no movements, even if it was applied vigorously enough to displace the animal in the dish.

Stimulation with visible light. These observations were the outcome of an attempt to employ the easiest method of restricting a beam of infra-red rays accurately to specified areas of integument. After the heat-absorbing filter had been exchanged for an infra-red filter, the lamp was switched on for a brief period. This allowed a beam of visible light normally excluded by the infra-red filter to be brought quickly on to the selected area. Illumination of the fin in the middle third of the animal evoked no movement, but as soon as the beam struck the nerve-cord, it invariably and immediately caused the animal to execute vigorous wriggling movements and to thrash around for from 15 sec to 2 min in the dish; the time depended upon the intensity of the illumination.

Stimulation with infra-red rays. In view of the effect of visible light, the infra-red rays were focused by sighting and measurement. A single freely suspended thermocouple (40 S.W.G. wire) was gently lowered on to the integument in the centre of the area selected for stimulation (a manœuvre which evoked no movements) and the infra-red generator positioned accordingly. The temperature of the integument was then recorded and the lamp switched on. Both the rise in surface temperature and the time which elapsed between the onset of stimulation and the onset of movement were noted.

Over 20 stimuli, ranging from transfers of 1·2 to 1·8 cal/cm^2/sec, were delivered in random order to each of the 5 animals. The total number of stimuli delivered was 170.

Part of a protocol from one of the experimental runs is given in table 3.

TABLE 3

	Strength of stimulus (cal/cm²/sec)	*Interval from previous stimulus (min)*	*Temperature (°C) of integument*		*Rise in temp (° C)*	*Movements exhibited*	*Duration of stimulus (sec)*
			Initial	*Final*			
1	1·8	First stimulus	13·5	18·0	4·5	Sprang out of water	8
2	1·8	11	13·5	16·0	2·5	,, ,,	5
3	1·2	10	14·0	17·0	3·0	Wriggled away from stimulus	20
4	1·8	13	13·5	18·5	5·0	Sprang out of water	12
5	1·5	17	13·5	17·0	3·5	Wriggled away from stimulus	11
6	1·2	10	14·0	19·0	5·0	Nil	60

Table 3 shows the effect of irradiation on specimen no. 18. Length, 5·1 cm. Temperature of water, 13·5° C. Site of irradiation, right postero-lateral middle third of animal.

It was clear from these experiments that the surface temperature of the integument in the area chosen rose by an amount which was dependent upon the intensity of the radiation. It was also found that the relationship between stimulus and movement was significant to a level $P > 0{\cdot}01$ provided that the rate of transfer exceeded 1·2 cal/cm²/sec. The actual threshold varied, however, from animal to animal and from time to time in the same animal.

In two of the animals in which the thresholds were consistently low, the effect of exposure to a low rate of heat transfer over a fairly long period was investigated. The rate used was below threshold and chosen arbitrarily to cause a rise in surface temperature of approximately 1° C per min. In the case of the first animal, the surface temperature at the centre of the area irradiated had risen by 10·5° C after 10 min but the animal had not moved. In the case of the second animal, the surface temperature at the centre of the area irradiated had risen by 11° C after 10 min, but it likewise had not moved. The first animal was similarly irradiated on three subsequent occasions and the second animal on five subsequent occasions at intervals of not less than 10 min. On no occasion were any movements evoked. After an interval of 2 h the first animal was again exposed to a low rate of heat transfer for 5 min and the temperature at the centre of the area irradiated rose by 5° C; the stimulus was then abruptly increased in strength to 1·2 cal/cm²/sec. Five seconds later, when the surface temperature had risen by a further 1° C, the animal suddenly started to wriggle vigorously around the dish. The second animal reacted in a comparable manner 3 sec after the stimulus had been abruptly increased in strength and the surface temperature at the centre of the area irradiated had risen by a further 0·55 to 0·75° C.

At the end of these experiments, both animals appeared to be just a responsive and appeared to move just as vigorously when stimulated b white light as they had before stimulation with infra-red rays. Twenty-fiv hours later, both animals were fixed in Bouin's fluid and cut into seria transverse sections, which were stained with haematoxylin and eosin an examined under the microscope. The appearance of the integument, in th areas which had been irradiated, was comparable in every way with that on th opposite side and with that in animals which had not been irradiated.

Operated animals (*in which the integument covering the dorso-lateral aspect o the middle and caudal thirds on one side had been removed*).

Control experiments. In each of 6 further animals which were similarl irradiated, first on one side and then on the other, with a stimulus of 1·8 cal cm^2/sec, the figures obtained fell within the range of those determine previously.

Stimulation of operated animals. Half an hour after operation, during whic time it had not changed its position, each animal was again irradiated o both sides (in random order) with a stimulus of 1·8 cal/cm^2/sec. Five animal were similarly irradiated for a second time after an interval of 2 h and tw of them for a third time an hour later. The results of the experiments in eac of the 6 animals are set out in Table 4.

TABLE 4

Animal	*Length in cm*	*Time of onset of movement in sec*	
		Normal side	*Operated side*
K	6	7	26
		6	25
		9	31
L	3·7	5	26
		7	36
M	5·3	13	20
		4·5	9
N	5·2	12·5	20
		11	29
O	3·5	10	35
		7	17
		9	26
P	5·7	31	50

Table 4 shows the response evoked by transferring heat at the rate of 1·8 cal/cm^2/sec t the dorsal surface of the middle third of amphioxus on the normal and operated sides.

The examination of serial transverse sections showed that the nerve-cor and the integument on the unoperated side had apparently suffered n damage and that no integument remained on the operated side.

These experiments favour the view that non-injurious infra-red stimul applied to normal animals are transduced by nerve-endings in the integumen as opposed to either the nerve-cord or the 'eye-cups' associated with it.

Discussion

In this series of animals, neighbouring epidermal cells covering the dorsal fin were indistinguishable in respect of their shape and staining properties. A number of authors, however, have reported seeing a few isolated (commonly darkly staining) cells with distinctive outlines in the epidermis (Joseph, 1908; Krause, 1921). They labelled them neuro-epithelial cells although they had no means of knowing whether or not axons arose from them. In this connexion, however, Franz (1923) voiced the view that in one sense every epithelial cell was a sensory cell, for nerves terminated at the base of each of them. By contrast, Dogiel (1903) explicitly states that he reserved the term neuro-epithelial for cells which not only stained darkly with methylene blue but appeared to fuse with nerve-fibres. He only came across such cells on very rare occasions. Thus, there is general agreement, which is supported by our observations, that specialized cells (presumably specialized receptors) are rare in the epidermis of amphioxus. In seeking to explain why the descriptions of neuro-epithelial cells which have been given lack precision, it occurred to us that the cells in question might have been ordinary epithelial cells but either injured or in a dying condition; dying cells do change in shape and in staining properties. For instance, they shrink and become deep blue before normal cells in tissues stained with methylene blue. Under such conditions they might well appear to fuse with blue-stained nerve-terminals which end in relation to every epidermal cell. There can be no proof of this suggestion, but because such cells are only seen sporadically it is a possibility which cannot be excluded in any discussion on the presence of neuro-epithelial cells in the epidermis of amphioxus.

The exact nature of the nuclei along the course of the nerve-trunks which combine to form the 'dorsal roots' of the nerve-cord has been a matter for dispute in the literature.

Some authors (e.g. Hatschek, 1892) have considered that they are the cell-bodies of the first sensory neurone. They argue that, in this respect, amphioxus is primitive and is displaying a characteristic which might have been expected. Most of its sensory nervous system has evolved beyond that of the neuro-epithelial stage towards, but has not yet reached, that acquired in vertebrates, i.e. a definitive dorsal root ganglion system (Young, 1954). Others are emphatic that there are no nerve-cells outside the nerve-cord in amphioxus (Heymans and Van der Stricht, 1898; also Retzius). It was suggested by Fusari (1889) that they were the nuclei of cells similar to those in the dura mater. Franz (1927), on the other hand, suggested that they were the nuclei of cells akin to glial cells. Those who rejected the hypothesis that they were the nuclei of the first sensory neurone (e.g. Heymans and Van der Stricht) pointed out that they can neither be seen clearly when techniques selective for nerve-cells and fibres are employed nor can processes be traced passing from the neighbourhood of the nuclei towards or away from the nerve-cord.

The evidence against the hypothesis that they are the nuclei of the firs sensory neurones is growing and now outweighs anything in its favour In the first place, we have shown that comparable nuclei can be seen alon the course of the nerve-trunks which spring from the 'anterior roots', whic are wholly motor in function in amphioxus. Secondly, no discrete cell-bodie envelop the nuclei and no processes can be seen passing from their immediat neighbourhood towards or away from the nerve-cord. Moreover, the nucle are not selectively stained by techniques which can be successfully used t demonstrate nerve-fibres and nerve-cells elsewhere. Thirdly, the nuclei which appear to contain no sharply defined nucleoli, are smaller than thos nerve-cells in the cord; their size is remarkably constant and their numbe along the course of the ramus dorsalis of the 'dorsal root' and the nerves whic subserve it far exceeds the number of parent sensory nerve-fibres which ca be impregnated with silver in this purely sensory trunk. In the light o Gasser's (1956) observations, it is possible to argue that the technique whic we employed did not impregnate all the axons in the nerve-trunk. On th other hand, the work of Ranson, Droegemueller, Davenport, and Fishe (1935) concerning the relationship between the number of dorsal-root gang lion cells and the number of dorsal-root axons which can be impregnate with silver in mammals suggests that any discrepancy encountered is unlikel to be of the order revealed by our counts, particularly in view of the hig ratio we found between the number of parent axons and the area of integu ment which they collectively served. Thus, the nuclei in question are probabl associated with the sheaths which accompany sensory nerves from the base ment membrane beneath the epidermis to the nerve-cord, and are therefor likely to be of the nature of the nuclei of the Schwann cells of vertebrates rather than the nuclei of nerve-cells.

Observations reported in this paper on the patterned arrangement of th nerves serving the integument elaborate upon previous descriptions in tw important respects. In the first place, the ratio of epidermal cells to paren axons serving them in randomly selected metameres of integument coverin the lateral aspect of the dorsal fin in the middle third of the trunk has bee estimated to be as low as 7:1. Moreover, the surface area in square mm divided by the number of dorsal roots serving it is 0·034. In man the smalles figure obtained in this way, which has been reported, is 0·65 (Ranson an co-workers); in the ear of a 3-week-old rabbit it is about 0·175 (figure calcu lated from Weddell, Pallie, and Palmer, 1955; Weddell and Pallie, 1955 Weddell, Taylor, and Williams, 1955). These figures are not, of course strictly comparable, and their full significance is unknown, but they d suggest that the density of innervation of the integument of amphioxus in th region of the dorsal fin can hardly be described as meagre. At first glance i certainly appears to be meagre in comparison with that of, say, the cornea but this is due to the fact that in amphioxus both the collaterals derived fro the parent axons and their terminal arborizations are extremely fine. In addi tion, the terminal arborizations are confined within a narrow zone which i

difficult to prepare for examination in the form of a continuous sheet of undamaged tissue, for the epidermis consists of a single layer of cells only, and what is more, the thickness of the integument over the dorsal fin is nowhere greater than 30 μ. Thus the only nerves to catch the eye are the bundles lying in the subcutis.

In the second place, a description has been given of the patterned arrangement of the collaterals and pre-terminal branches of parent sensory nerves serving the integument, together with an account of the relationships one to another of the terminals derived from parent axons of different diameters. The examination of successive metameres suggests that these relationships are entirely random although the large number of, and complex pathways pursued by, the fine axons forming the sub-epithelial plexus, make it impossible to be dogmatic on this point. Thus, despite the simple nature of the integument in amphioxus, the patterned arrangement of the nerves serving it is comparable with that in the skin of teleost fish (Whitear, 1952) and the cornea of a number of vertebrate species (Zander and Weddell, 1951*a*). (Incidentally, the latter comparison was foreshadowed by Zelinka as long ago as 1882.)

Numerous factors determine the characteristic patterned arrangement of the axons and terminals in the integument of lower vertebrates. Among other things, Weiss (1950) cites experimental evidence demonstrating the influence of (1) differences and changes in metabolic equilibrium on the specific growth potential possessed by primary sensory neuroblasts; (2) the specific properties of the tissues through which the tips of their growing processes come to pass; (3) the influence of the growing processes of other neurones which terminate within the same area of integument; and (4) the particular nature of the goal towards which the processes are advancing.

Additional evidence that the final pattern displayed by nerve-fibres in integument is not a matter of pure chance is suggested by the events which take place in the cornea after keratotomy. Zander and Weddell (1951*b*) have shown that the denervated zone is rapidly re-innervated by sprouts which grow in from surrounding undamaged axons. Remodelling (by successive waves of invasion and degeneration of axons) then takes place until all the severed nerves that are not permanently obstructed have given rise to new processes which re-enter and terminate within the cornea to form a pattern comparable with that seen on the unoperated side.

The fact that a series of morphological readjustments of this kind consistently take place in the cornea after keratotomy might be used to support the generalization of Sperry (1950) that peripheral sensory neurones are each endowed with specificities of a highly refined nature. Further, the close similarity between the arrangement of the nerves in the integument of amphioxus and of those in the cornea make it not unreasonable to suppose that each of the sensory neurones serving the integument in each of the metameres in amphioxus is endowed with a high degree of refined specificity, but there is, of course, no proof either that this suggestion is correct or that,

if correct, it is of any significance in the reaction the animal displays to stimul received in the course of daily life.

Our observations did not establish the precise mode and place of termination of the nerves serving the integument. In well-fixed preparations the nerves appear to end by attenuation at the base of the epidermal cells as described by Retzius, Franz, and others. By contrast, in fresh (but dying) preparations, filaments can be seen passing between epithelial cells towards the cuticle, beneath which they appear to end blindly, observations more in accord with those of Dogiel. Clearly, the solution of this problem lies in the successful application of methods of microscopy capable of yielding higher magnifications without loss of resolution. Preliminary observations made by Whitear (1957) with the electron microscope indicate that some of the nerve-fibres in the corneal epithelium are situated in deep grooves on the base of epithelial cells. Indeed, casual observation suggests that they are lying within the cytoplasm of the cell-bodies, whereas in fact they invaginate the walls of the cells and remain at all times extracellular.

Parker's (1908) experimental observations have been amplified by the demonstration that nerves in the integument of amphioxus can transduce non-injurious stimuli resulting from heat transfer by irradiation (at a rate of 1·2 millical/cm^2/sec or more) into nervous activity sufficient to cause displacement of the animal by undulating movements involving the whole trunk.

Some of the stimuli chosen by Parker for application to the integument had physical attributes equivalent to those which (by common consent), when delivered to the skin in man, evoke reports of touch, warmth, cold, and pain. Most of the other stimuli he chose (solutions of chemical substances) would evoke reports of pain if applied to exposed mucous membranes in man. In addition, he observed the behaviour of animals in the tanks in which they were living and noted in particular that amphioxus is very sensitive to sound. Even the noise Parker made on entering the darkened tank room as quietly as he could caused the animals to wriggle more deeply into the sand. He advanced evidence in favour of the view that noise was effective because it gave rise to vibration and hence mechanical stimuli which were transduced by nerve-terminals in the integument. We have confirmed Parker's observations in relation to animals living in a tank room but we failed to evoke any significant reactions to sound from animals which were lying in a glass-bottomed dish.

Franz (1924) also reported an interesting observation in relation to the stimulation of relatively large areas of integument. He noted that the mechanical stimulus which invariably invoked the most vigorous and prolonged outbursts of movement was a brushing contact of the integument anywhere over the body-surface delivered by another amphioxus swimming in the immediate neighbourhood.

Ten Cate (1938*b*) was the first to draw critical attention to the artificial nature of the experimental procedures employed by many workers in this

field. For example, he demonstrated that a standard mechanical stimulus, such as a needle prick, was far more effective when applied to animals which had been permitted to bury their bodies in the sand than it was when similarly applied to animals isolated in glass-bottomed dishes. He also demonstrated, in relation to needle pricks, that the nervous system in amphioxus is so organized that the only response which can be evoked is displacement forwards or backwards (away from the stimulus) resulting from co-ordinated undulatory movements involving the musculature throughout the body. This reaction, as he pointed out, is closely related to the particular needs of amphioxus in the environment in which it lives. In other words, in the case of amphioxus (as opposed to man), in its normal habitat, the effectiveness of a needle as a stimulus object is related both to the fact that it pierces the integument locally and the fact that it gives rise concomitantly to a series of punctate stimuli over a wide area of integument by grains of sand tending to resist the displacement of the animal by the needle.

In this connexion, Rohon (1882) and Kohl (1890) deduced that light was not an effective stimulus; but their experiments were carried out on amphioxus almost completely buried in the sand, as they are normally in nature. In both Krause's (1897) and our own experiments there was no doubt about the effectiveness of light stimuli, but in both cases the animal was completely uncovered in a flat-bottomed dish so that all the eye-cups in the cord were exposed, a condition which occurs but rarely in nature (Ten Cate, 1938*b*).

From the evidence available, then, it appears that the gross motor reactions of amphioxus to stimulation are most easily explicable on the basis that they are dependent upon the site of origin as well as the number and patterned arrangement of the nerve impulses which reach the cord; they are not necessarily dependent upon the immediate physical attributes of the stimulus which evokes them. As Ten Cate has already pointed out, to make a really critical assessment of the organization of the mechanisms underlying the functional activity of the nervous system, it is necessary to study further the pattern of the reactions of amphioxus to the changing environmental conditions in its natural habitat. A study confined to noting its reactions to stimuli which obviously link man with the changes in his own environment may be very misleading.

When considered from this point of view, it is clear that in its normal habitat amphioxus is unlikely to receive stimuli having physical attributes similar to some of those employed by Parker. The choice of radiant heat as a stimulus to be delivered to a small area of integument in the region of the dorsal fin of an animal isolated in a glass dish was clearly in the nature of an academic exercise and far more unnatural than any of the experiments carried out by Parker. However, the results of these experiments demonstrate quite unequivocally that free nerve-endings in the integument of amphioxus can transduce non-injurious stimuli having physical attributes which are of a kind not encountered in the natural habitat of the animal.

It is of some interest that unencapsulated nerve-endings in the integu ment (though apparently not in the case of amphioxus) can, under certai conditions, transduce light stimuli. For instance, Parker (1903) advance experimental evidence for the view that light can be transduced by nerve endings of spinal origin in the skin of the frog. In the case of the lamprey ther is experimental evidence that unencapsulated nerve-terminals in the skin o the tail transduce light stimuli (Young, 1935). However, the nerves servin these particular terminals are of cranial origin and the terminals themselve are related to epidermal cells which appear to be specialized to assist in th transducing of light stimuli (Steven, 1951). Such observations serve to add t the number of stimuli with different physical attributes which unencapsulate nerve-terminals in the integument of different animals are capable of trans ducing. They also make it clear that certain unencapsulated nerve-terminal (morphologically indistinguishable from their neighbours) in the integumen are in some way differentiated to transduce stimuli having specific attributes Thus it is possible to argue that some terminals in the integument of am phioxus may have a low threshold to either positive or negative heat transfer as Parker originally claimed. However, our experimental observations i relation to the effectiveness of unnatural stimuli suggest that it is mos necessary to remember that *functional* specificity may only apply in relatio to one or more of the range of stimuli which normally impinge on a specifi zone of integument covering a particular species of animal living in surround ings that are natural to it.

Precise information as to the specificity of nerve-terminals in the integu ment of amphioxus in relation to stimuli having physical attributes likely t be encountered in its normal habitat can only be obtained directly by record ing from single sensory nerve-fibres—technically a difficult task. Moreover, i will not be sufficient to record from single parent axons serving a given area o integument at random. The activity in each and every axon serving the are will have to be recorded in relation to each stimulus object, for Lele (1954 has shown that in human skin free nerve-endings are not related to therma sensibility in a direct and simple way. Indeed, his work forces one to th conclusion that the temporo-spatial pattern of activity reaching the centra nervous system must be a factor of some importance in relation to modalit discrimination. This in turn indicates that the patterned arrangement o nerve-fibres and their terminals in the skin is also of importance in thi respect. There is thus every reason to suppose that the complex patterne arrangement of nerve-fibres and endings in the integument of amphioxus which in many respects resembles that in the skin of man very closely, may in addition to the possible existence of 'modality specific' nerve-terminals play a part in determining the response evoked by a given stimulus object

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